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Genetic differentiation of a sympatric lake-resident/anadromous species pair of threespine stickleback in Mud Lake, Alaska

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Genetic Differentiation of a Sympatric Lake-Resident/Anadromous Species Pair of Threespine Stickleback in Mud Lake, Alaska

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ABSTRACT

The formation of new species is responsible for all the biological diversity we see on Earth. This process of speciation is not yet fully understood and is most often studied by comparing individuals of closely related species. The threespine stickleback, *Gasterosteus aculeatus*, a fish species complex found in marine and coastal freshwater environments of the northern hemisphere, has become a model species used to study speciation. Many morphological and genetically divergent forms within this complex have adapted to freshwater conditions. Studying the mechanisms that allow stickleback to adapt to freshwater conditions has provided much insight into the process of speciation. Here, we present the first genetic analysis of a lake-resident/anadromous species pair of threespine stickleback. This analysis is preformed on a lake-resident/anadromous pair found in Mud Lake, a freshwater lake in the Jim Creek drainage of Alaska. Anadromous and resident freshwater stickleback in Mud Lake differ substantially in morphology, including body size, body shape, armor, and trophic morphology. Based on previous morphological research, this species pair is believed to be breeding sympatrically with little evidence of hybridization. Seven additional lake, stream and anadromous populations from the Jim Creek drainage and neighboring drainages were included for comparison. Analysis of genetic diversity and tests of differentiation from a survey of neutral genomic markers (microsatellites) and DNA sequences from the mitochondrial control region indicate significant genetic divergence between sympatric anadromous and resident freshwater fish in the Jim Creek drainage. Genetic diversity was greater in anadromous populations than resident freshwater populations, consistent with previous genetic analyses of stickleback from other areas. Neighbor-joining trees based on genetic distances among populations revealed that resident freshwater populations in the Jim Creek drainage clustered together, forming a separate

cluster from the anadromous populations with which they breed sympatrically. Similarly, Bayesian cluster analysis revealed four genetic clusters: one composed of the anadromous populations, and three composed of resident freshwater groups segregated by geographic region, with the Jim Creek resident populations falling in one discrete cluster. Overall, the genetic data indicate a primary axis of genetic divergence corresponding to anadromous vs. resident freshwater populations, with the latter secondarily diverging by geographic location. Consistent with previous morphological research, we found little evidence that significant hybridization between the two forms is occurring. The similarity in the magnitude of genetic divergence between anadromous populations and resident freshwater populations breeding sympatrically in the Jim Creek drainage, compared to the divergence between anadromous populations and neighboring resident freshwater populations living in allopatry supported our hypothesis that these forms are reproductively isolated. They have likely been isolated for a period of time similar to that of other resident freshwater populations in the region. These results improve our understanding of the relationship of the species pair found in Mud Lake, and will provide a genetic baseline for research into the early mechanisms that support the formation of new species.

INTRODUCTION

The diversity of life we see on Earth can be directly attributed to the adaptive mechanisms that allow species to evolve to new forms. Although the way new species form is still not yet well understood, three processes are believed to be responsible for much of the adaptive divergence we see in nature: 1) divergent natural selection in which selection pressures favor distinct combinations of traits in different forms; 2) the evolution of reproduction isolation, and 3) ecological character displacement wherein competition for resources drives the divergence to exploit new resources (Schluter 1993). These processes are most often studied by comparing members of closely related species and drawing inferences about the mechanisms that facilitated the formation of these species (McPhail 1994). Though speciation is often considered part of the past, new species must currently be forming. If identifiable, the newly forming species provide the greatest opportunity to learn about the genetic, ecological, physiological and behavioral changes that take place during the formation of species (McPhail 1994).

The threespine stickleback (*Gasterosteus aculeatus*) is a native fish species complex (many morphologically divergent forms of the same species) found in temperate climate regions of the northern hemisphere. This species complex consists of thousands of subpopulations inhabiting a large biogeographical region and occupying a number of habitats, from coastal marine waters to freshwater lakes and streams (Bell & Foster 1994). The variation in stickleback behavior, morphology and life history, and the recent availability of certain genomic tools (Kingsley & Peichel 2007) make the threespine stickleback an excellent model for evolutionary and ecological studies (Bell & Foster 1994).

In the Cook Inlet region of Alaska (Figure 1A), there is an apparent species pair of threespine stickleback consisting of genetically and phenotypically divergent anadromous (ocean-run) and lake-resident forms that breed sympatrically in a small, postglacial lake called Mud Lake (Figure 2) (Karve *et al.* 2007). The anadromous forms live in the ocean and migrate to the freshwater lake to breed, while the lake-resident forms live in Mud Lake year round. The parallel evolution of stickleback species pairs, such as the ones found in Mud Lake, have contributed valuable insight into how new species evolve by divergent natural selection (McKinnon & Rundle 2002). The occurrence of lake-resident and anadromous type stickleback breeding sympatrically appears to be relatively rare, having only been reported in a small number of studies (see Ziuganov *et al.* 1987, Mori 1990, Higuchi *et al.* 1996, Kitano *et al.* 2003, von Hipple & Weigner 2004). Most anadromous/resident freshwater stickleback species pairs occur in streams (e.g., Hagen 1967; McPhail 1994). Based on previous research on Mud Lake, the species pair in question breeds sympatrically with little to no apparent hybridization (Karve *et al.* 2007; Bell *et al.* 2010). Mud Lake is part of a small drainage system that includes four lakes connected by Jim Creek (Figure 1D). The system drains into the Knik River. The distribution of resident freshwater and anadromous stickleback throughout the system is unknown. While the sympatric breeding of anadromous and lake-resident stickleback has only been reported in Mud Lake, it may occur in other lakes within the system.

This study examines the genetic relationships between the stickleback species pair found in Mud Lake and neighboring lakes in the Jim Creek drainage. Using neutral molecular markers from genomic and mitochondrial DNA, the magnitude of genetic relatedness was determined by examining genetic distances among the sympatric species and contrasting the distances with

those between other stickleback populations in the area. Trees depicting genetic relationships among populations were constructed and genetic structure was determined and analyzed across forms and geographic locations. Genetic diversity was also measured and contrasted between forms. This study presents the first examination of genetic relationships of this rare species pair and establishes a genetic baseline for future research on the isolating mechanisms at play in this system and during the early stages of speciation.

BACKGROUND

Speciation

One of the most fundamental problems in biology is the origin of species. Introduced by Charles Darwin (1859), the book *On the Origin of Species* presented the scientific theory that populations evolve over generations through a process known as natural selection. Although species are of central importance in biology, it is often difficult to define a biological species. While there are many species concepts, the biological species concept (BSC) is most prominently used in the classification of new species (Coyne & Orr 2004). The BSC defines species as groups of interbreeding natural populations that are reproductively isolated from one another (Mayr 1995). The BSC in not always perfect when applied in nature, like any concept that attempts to define a species, none can be totally free of ambiguities. For instance, allopatric populations tend to be difficult to classify because it is usually unknown whether their differences would allow them two live sympatrically without the exchange of genes. Fossils also pose a challenge, since the level of reproductive isolation in extinct populations cannot be determined. Furthermore, using the BSC to describe taxa that undergo full or partial asexual reproduction, such as bacteria, is very problematic. The complexity of the evolutionary process will often produce unclear cases that would be difficult to classify by any set of definitions (Hey 2001), even "good" species can often form viable hybrids in nature. Yet with its limitations, the BSC allows researchers to study the process of speciation by helping them understand how discrete interbreeding populations arise.

The evolution of reproductive isolation is a major factor in the process of speciation. There are many mechanisms that can lead to the evolution of reproductive isolation. These can

be classified into two major groups, prezygotic and postzygotic. Prezygotic isolation occurs when barriers inhibit gene flow before the transfer of sperm to members of other species (Coyne & Orr 2004). This can develop when differences in behavior or ecology prevent different species from initiating courtship or copulation. Mechanical isolation can also occur, in which copulation is inhibited due to incompatibility of reproductive structures. The barriers of postzygotic isolation can best be described as hybrid sterility and inviability. Hybrid sterility occurs when hybrid offspring incur problems that inhibit the normal development of the reproductive system, gametes or other physiological functions, impeding future reproduction. Hybrid inviability takes place when hybrid offspring develop normally but suffer from reduced ecological fitness due to the inability to find a niche, or develop intermediate behavioral characteristics that inhibit their ability to find mates (Coyne & Orr 2004).

There are two major modes of speciation that are generally recognized. First, allopatric speciation occurs when reproductive isolation evolves after the splitting of a species' geographical range, forming two or more isolated populations. Over time, the isolated populations develop prezygotic and/or postzygotic barriers that would inhibit reproduction should the populations ever come in contact (Coyne & Orr 2004). In contrast, sympatric speciation involves the evolution of new species from an ancestral type while each occupies the same geographical range (Mayr 1963). Sympatric speciation is thought to be less common than allopatric speciation because of two fundamental problems (Coyne & Orr 2004). First, disruptive selection resulting in the split of sympatrically breeding populations would likely be countered by interbreeding that breaks down co-evolving gene complexes responsible for reproductive isolation. Thus, sympatric populations must develop mechanisms to inhibit

recombination in order to evolve reproductive isolation. Second, species evolving in sympatry must diverge ecologically in order to coexist during and after the evolution of reproductive isolation. That is, populations must overcome competitive exclusion in order to exist. Whichever processes are acting on natural populations, the evolution of reproductive isolation ultimately results in the formation of new species.

Organism of Study – Threespine Stickleback (*Gasterosteus aculeatus)*

The threespine stickleback represents a hyper-variable species in the family Gasterosteidae (order Gasterosteiformes) that can best be described as a species complex (Bell & Foster 1994). This species complex consists of thousands of morphologically divergent populations distributed throughout temperate regions of the Northern Hemisphere (Figure 3). Stickleback occur in a variety of habitats within coastal marine waters and freshwater lakes and streams, and can range in size from 5 to 11 cm from tip of the snout to the end of the vertebral column (Bell & Foster 1994). Primitively, stickleback are oceanic, but the stickleback's ability to adapt to a vast array of aquatic habitats, from brackish to fresh waters, accounts for its expansive geographical distribution. It is because of this adaptive ability that the threespine stickleback exhibits rapid evolution of morphology, behavior and physiological traits. Behavioral research dating back to the 1950's (e.g., Tinbergen 1951) helped bring attention to the threespine stickleback. Since then, an enormous amount of research has been carried out on the behavior, ecology and evolution of the threespine stickleback (reviewed in Wootton 1984; Bell & Foster 1994; McKinnon & Rundle 2002; Ostland-Nilsson *et al.* 2007; Hendry *et al.* 2009). In 2006, the first draft assembly of the stickleback genome was completed by the Broad Institute at MIT and Harvard (Broad Institute v1.0), increasing the value of this species as a

model organism by allowing researchers to peer into the stickleback's genetic architecture to understand the molecular basis of vertebral evolution.

Stickleback Adaptive Radiation and Speciation

At the end of the last ice age, the melting of ice caused sea levels to increase more than 100 m (Mann 1986). Glacial flooding of coastal areas or migration inland may have contributed to the colonization of freshwater regions by oceanic stickleback (McPhail & Lindsay 1986). The invasion of freshwater streams and lakes by oceanic populations of stickleback has resulted in thousands of phenotypically and genetically divergent populations (Figure 4). This rapid development of diverse forms adapted to local conditions is known as adaptive radiation (Schluter 2000). In some instances, stickleback populations have diverged so much so that they behave like distinct biological species (reviewed in McPhail 1994, Hendry *et al.* 2009).

Much like the famous models of adaptive radiations—the African cichlids with their elaborate color morphs and trophic diversity (Fryer & Iles 1972) and Darwin's Galapagos finches with their divergent beaks (Grant & Grant 2008)—stickleback populations adapted to different habitats have undergone many phenotypic changes, including changes in lateral armor plating, the pelvic girdle, trophic morphology, and body shape, as documented by Bell & Ortí (1994); Walker (1997); Bell *et al.* (2004) and Aguirre (2009). Furthermore, major morphological changes like shifts in lateral plate phenotypes can occur within a few generations of freshwater colonization (Bell *et al.* 2004). Studying these morphological and genetic differences can help develop hypotheses about how mechanisms for reproductive isolation evolve and how the early stages of speciation proceed (Bell & Foster 1994).

It often proves difficult to identify the genetic or morphological changes that occur prior to speciation. Unfortunately, the progression of speciation is usually identifiable only after the events have occurred and organisms behave like biological species. Closely related species pairs that occur sympatrically but remain reproductively isolated provide some of the best opportunities to study the mechanisms of speciation (McPhail 1994). The majority of studies of speciation analyze these closely related species or populations, such as African cichlids, to make inferences on how the mechanisms of reproductive isolation have occurred (e.g., Martin & Genner 2009). Some of the most extensive research on speciation has been carried out on the threespine stickleback. McPhail (1994), McKinnon and Rundle (2002) and Hendry *et al.* (2009) have reviewed a variety of species pair types of the threespine stickleback and speculated on the mechanisms of reproduction isolation including: temporal, spatial, and behavioral isolation, and hybrid inferiority. Stickleback species pairs will often differ depending on when and where they breed and who they choose to mate with, all of which likely contribute in part to reproductive isolation. Furthermore, hybrids between species pair types may exhibit lower fitness because they contain a mixture of traits adapted to separate environments. These previously studied species pairs include: lake/stream, benthic/limnetic, red resident/black resident, typical marine/white marine, Japan Pacific/Sea of Japan, and anadromous/resident freshwater.

Molecular Markers and Measuring Genetic Divergence

Molecular markers provide information about species and populations at the genetic level. Prior to the use of molecular markers, researchers were limited to studying genetics and heredity through the observation of phenotypic variation among kin. However, phenotypic studies of genetic variation are very limited in their power to help us understand the wealth of

genetic diversity that exists on Earth. The use of molecular markers allows researchers to directly examine genetic variation, and to analyze inter- and intra-specific relationships (Avise 2004). The molecular revolution began when researchers developed the methods to conduct allozyme studies in the mid 1960's (reviewed in Avise 1974). Allozymes provided the first data on levels of molecular variation and divergence among organisms in nature. However, allozymes are limited in their usefulness because as markers of protein variation they are only indirect markers of genetic variation. As a consequence, much of the genetic variation among organisms remains hidden because DNA level changes that do not affect proteins cannot be measured. With the introduction of the polymerase chain reaction (PCR), allozyme techniques were largely replaced by more powerful methods, such as direct DNA sequencing and the analysis of microsatellite loci, that allow direct scoring of DNA-level variation (reviewed in Avise 2004).

Since the introduction of a DNA sequencing technique by Sanger *et al.* (1977), genetic analysis of raw sequence data has become a valuable tool for studying genetic variation within or among populations (Kocher *et al*. 1989). Mitochondrial DNA analysis became a very popular tool in the 1970's and 1980's for conceptual and practical reasons (reviewed in Avise 2004) and is still popular today. Mitochondrial genes are maternally inherited, which results in a smaller effective population size (number of individuals that would result in the same loss of genetic diversity under random genetic drift or the same amount of inbreeding as an idealized population) and a higher rate of evolution compared to nuclear genes, due to a higher nucleotide substitution rate (Avise 2004). Recent studies using the North American mountain goat (Shafer *et al.* 2011), Lake Malawai rock cichlids (Genner *et al.* 2010), European nine-spined stickleback

(Shikano *et al.* 2010) and threespine stickleback (Berner *et al.* 2010) continue to affirm the approach of comparing mitochondrial sequences as a valuable tool to answer numerous population-level questions. Most of the mitochondrial genome codes for proteins or tRNA. The mitochondrial control region or d-loop is a non-coding portion of the mitochondrial genome that has been useful in population-level studies because of the high rate of mutation from frequent nucleotide substitutions (Palumbi 1994). Because the mitochondrial genome is only transmitted along female lines, it provides a useful complement to nuclear markers for understanding the evolutionary history of populations (e.g., Shikano *et al.* 2010). The control region has traditionally been difficult to amplify in the threespine stickleback. However, robust primers have recently been developed that seem to consistently amplify a small fragment of the control region in threespine stickleback. This development enables a more widespread use in population genetic studies (Mäkinen & Merilä 2008).

Microsatellite loci (or "short tandem repeat" loci) are variable-number repeating segments of DNA (e.g., ACACACACAC…) that typically emerge in non-coding regions of the nuclear genome and exhibit co-dominance (both alleles can be scored unambiguously). Microsatellites are popular in studies of closely related organisms, like studies of kinship relationships and the genetic structure of populations (e.g., Berner *et al.* 2010; Shafer *et al.* 2010) because of their high mutation rates, abundance within the genome and the large number of alleles observed in most natural populations (Jarne & Lagoda 1996). Microsatellites have been used in a number of studies examining genetic variation of threespine stickleback populations. Population level studies such as those by Taylor and McPhail (2000), Reusche *et al.* (2001) and Makinen *et al.* (2006) have shown that using microsatellites is an effective way to determine

genetic relationships and quantify genetic variation in stickleback, even for populations that have diverged recently, like post-glacial populations.

The Mud Lake System

Mud Lake is a small freshwater lake occurring in the lowlands between the Matanuska and Knik Rivers approximately 62 km northeast of Anchorage, Alaska (Figure 1). Mud Lake is connected to the Knik River via Jim Creek and has an area of 1.73 km^2 (Alaska Department of Fish and Game, unpublished data). Neighboring Gull Lake, Jim Lake and Swan Lake lie between Mud Lake and the Knik River; however, they may represent a continuous connected system of lakes as observed by satellite imagery (Google Earth). From October to April, lakes in this area are typically covered with ice (Woods 1985).

This study concentrated on two forms of stickleback that have been reported to breed sympatrically in Mud Lake, Alaska (Figure 2): anadromous (ancestral oceanic type that enter freshwater lakes and streams to breed) and resident freshwater. While both forms regularly inhabit lowland streams throughout much of the stickleback's geographic distribution (McPhail 1994), there are few published reports of anadromous/resident freshwater stickleback species pairs in lakes. They have been reported in the Azabachije basin in Kamchatka (Ziuganov *et al.* 1987), Lake Sana in the Kuril Islands (Mori 1990), several lakes on Hokaido Island (Higuchi *et al.* 1996, Kitano *et al.* 2003), new lakes near the Bering Glacier in Alaska (von Hipple & Weigner 2004), and Middleton Island, Alaska (Gelmond *et al.* 2009). Typically, any stickleback present in lakes are the resident freshwater form. In Mud Lake, however, both resident threespine stickleback and anadromous stickleback are known to be present during the breeding season

(May-July) (Karve *et al.* 2007). Three hypothesized colonization pathways may have led to the presence of this rare type of species pair in Mud Lake: 1) sympatric speciation from an anadromous ancestor, 2) invasion of anadromous stickleback into the lake in which they became isolated for a lengthy time interval due to barriers terminating lake access, or 3) colonization by resident stickleback from a separate freshwater system before or after a colonization of anadromous stickleback had occurred (Karve *et al.* 2007). Sympatric speciation is thought to be much more difficult than allopatric speciation due to the ability of sympatric populations to exchange genes freely (Coyne & Orr 2004); thus, Mud Lake residents most likely evolved from anadromous ancestors in allopatry, but this cannot be confirmed. Anadromous fish probably evolved into resident fish after becoming isolated in Mud Lake for a lengthy period of time, or anadromous fish evolved into resident freshwater stickleback elsewhere and migrated into Mud Lake before anadromous fish began running into the lake or after the anadromous population was already established.

Karve *et al.* (2007) showed that the two forms of stickleback that inhabit Mud Lake differ in body size and shape, fin size and lateral plate morphology (Figure 2). The anadromous population closely resembles other anadromous and marine populations in the region in being relatively large, having large eyes, many gill rakers to exploit a marine diet of plankton (McPhail 1984), a complete series of approximately 33 large armor plates covering the entire flank of the body, relatively large dorsal and pelvic spines, and large pectoral fins used in spring migrations (Aguirre *et al*. 2008). The resident stickleback population in Mud Lake is significantly smaller in size and morphologically resembles typical resident freshwater populations in the region. Perhaps most notably, it lacks the complete series of armor plates along the flanks that

anadromous populations possess, having less than ten plates restricted to the anterior part of the body instead. The resident population inhabiting Mud Lake appears to be adapted to a benthic lifestyle (McPhail 1984; Walker 1997); it is relatively deep bodied and has fewer gill rakers than many other resident populations in the region. Despite breeding sympatrically in Mud Lake, the resident and anadromous forms appear not to hybridize based on indirect data. Karve *et al.* (2007) surveyed a large number of resident and anadromous individuals collected throughout the lake and found no morphologically intermediates, which occur in hybrid zones documented in streams (e.g., Hagen 1967). A survey of the locus primarily responsible for lateral armor plating in stickleback, the *Ectodysplasin* (*Eda*) locus, also indicated no evidence of gene flow (Bell *et al* 2010). Bell *et al.* (2010) found that anadromous fish in Mud Lake were entirely homozygous for the complete-morph *Eda* allele. Since low-morph fish contain two recessive alleles, this indicates that the low-morph residents in Mud Lake are not integrating their low-morph alleles into anadromous fish. The apparent lack of hybridization may be due to factors related to physical traits such as size and/or breeding habits (Karve *et al.* 2007). Karve *et al.* (2007) found that males of the two forms display breeding coloration at the same time, but the number of resident males in breeding coloration appears to peak approximately five weeks before anadromous males. Also, the proportion of each type caught during the breeding season varied significantly by location, pointing to a potential preference for different nesting sites. Although the two types showed some divergence in spatial and temporal preferences, Karve *et al.* (2007) found broad overlap among individuals of the two forms, indicating that there is ample opportunity to hybridize.

This study seeks to expand our understanding of the relationship between the two stickleback populations inhabiting Mud Lake. No other study has examined the genetic relationship between a sympatric lake-resident/anadromous species pair of threespine stickleback. Microsatellites and mitochondrial control region sequences were employed to examine the extent of genetic differentiation between anadromous and resident freshwater populations inhabiting Mud Lake. The magnitude of genetic relatedness of the Mud Lake species pair will be compared to other populations of anadromous and resident freshwater stickleback in the region. Additionally, neighboring lakes in the Jim Creek drainage, to which Mud Lake belongs, were surveyed for the occurrence of sympatric anadromous and lake-resident stickleback populations.

Based on the magnitude of the morphological divergence between resident freshwater and anadromous stickleback in the system and previous stickleback research, a series of predictions can be made. 1) Anadromous and resident freshwater stickleback occurring sympatrically in Mud Lake and neighboring lakes in the Jim Creek drainage will differ significantly from one another at neutral molecular markers; 2) Resident freshwater forms within the system will form a genetic cluster that is distinct from anadromous stickleback samples collected in the system; 3) There will be little evidence of hybridization between sympatric resident freshwater and anadromous stickleback breeding sympatrically in this system; 4) The magnitude of genetic divergence between anadromous and resident forms breeding sympatrically in this system will be comparable to that between anadromous and resident freshwater forms that breed allopatrically in neighboring drainages.

METHODS

Sampling

A total of 600 threespine stickleback specimens representing 10 populations were collected from the Cook Inlet region of Alaska (Figure 1). Nine populations were sampled in June 2010, and one sample collected in June 2003 was also included as a reference sample (Table 1). Sample sites include three sites in the Jim Creek drainage (Figure 1D) from which resident stickleback were collected: Mud Lake, Gull Lake, and Jim Lake. Samples were collected twice at Mud Lake, once in June 2003 and once in June 2010. Anadromous stickleback were found to occur throughout much of the Jim Creek drainage, and samples of anadromous stickleback from Mud Lake and Jim Lake were also collected. Samples of four other resident freshwater and anadromous populations were collected from neighboring drainages in the Mat-Su Valley and the Kenai Peninsula to serve for genetic comparisons to the experimental populations (Table 1, Figure 1). Unbaited minnow traps set overnight were used to collect all specimens. Fish were euthanized using a lethal dose of MS-222 and immediately preserved in 95% ethanol.

DNA Isolation

Caudal fin clips were removed from 48 specimens per population and placed in a solution of tissue digestion buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 10mM EDTA, 0.5% SDS) and proteinase K (20mg/ml). Fin clip tissue was incubated overnight at 55°C. After incubation, 600 µl of 25:24:1 phenol:chloroform:isoamyl alcohol was added and the solution was centrifuged at 12,200 rpm for ten minutes to isolate the DNA. DNA was then washed with 100% ethanol and 70% ethanol. Concentrated DNA was re-suspended in 100 µl of TE buffer and stored at -80°C.

Working stock DNA was created by diluting the concentrated DNA in nuclease-free, molecular biology-grade water to concentrations of approximately 30 ng/μl of DNA (measured with a NanoDrop 2000C spectrophotometer) and stored at -20°C.

Microsatellite Amplification and Scoring

Nine Microsatellite loci from the nuclear genome of the threespine stickleback were amplified for 48 specimens per population (Table 2). Primers were chosen from those available in (Largiader *et al.* 1999) and (Peichel *et al.* 2001). The nine primers were chosen to represent a wide coverage of the nuclear genome (e.g., correspond to different chromosomes). Microsatellite loci from linkage groups (LG) defined by Peichel *et al.* (2001) that contained genes of large effect (e.g., *Eda* and *Pitx1*) previously identified by QTL mapping (Peichel *et al.* 2004; Colosimo *et al.* 2005; Shapiro *et al.* 2006), were avoided due to their potential for influencing the evolutionary history of nearby neutral markers. A QIAGEN Multiplex PCR kit (Cat. # 206143) and fluorescently labeled primers were used to amplify the microsatellite loci in panels of three. The composition of the panels, linkage groups, size range of individual loci and dyes used are presented in Table 2. The concentrations of reagents for the PCR reactions were as follows: 3.75 μl Multiplex PCR Master Mix, 0.75 μl Q solution, 0.15 μl 10 μM primer [3 fluorescently labeled forward primers + 3 reverse primers per panel], 1.35 μl water and approximately 20-25 ng DNA for a final volume of 7.5 μl per specimen. PCR conditions consisted of a single cycle at 95°C for 1 min 45 s, 56°C for 45 s and 72°C for 45 s; followed by 4 cycles of 94°C for 45 s, 56°C for 45 s and 72 for 45°C; continuing with 30 cycles of 90°C for 45 s, 56°C for 45 s and 72°C for 45 s; and a final extension at 72°C for 7 min. PCR products were diluted to 20 μl with nuclease-free, molecular biology-grade water and sent to the University of

Arizona's Fragment Analysis Facility for processing (http://uagc.arl.arizona.edu/index.php/fragment-analysis.html). Microsatellite loci were scored, binned and checked manually for scoring errors using GENEMARKER (SoftGenetics). Loci were compiled into an electronic spreadsheet and file conversions were done using CONVERT (Glaubitz 2004) and Genepop 4.0 (Raymond & Rousset 1995). MICRO-CHECKER (Van Oosterhout *et al.* 2004) was used to assess the quality of microsatellite data by comparing loci to Hardy-Weinberg expectations. Three markers (Stn195, Stn120, 4170PBBE) were found to be consistently heterozygous deficient across most populations. Due to the possibility of null alleles at these loci and their potential to erroneously steer results, the analyses were done with and without these loci. Their inclusion did not materially influence any conclusion; therefore, all analyses include the three loci.

Sequencing the Mitochondrial Control Region

Based on the recent design of primers that successfully amplify a 450 bp segment of the *G. aculeatus* mitochondrial control region (Mäkinen & Merilä 2008), this highly variable region was used to compare a subset of six of the ten samples included in the analysis of microsatellite loci. Twenty-four fish from each of two resident freshwater populations (Mud Lake Resident, Jim Lake Resident) and two anadromous populations (Mud Lake Anadromous, Jim Lake Anadromous) from the Jim Creek drainage were used. Twenty-four fish from each of two populations located outside the Jim Creek drainage (Wasilla Lake resident and Rabbit Slough anadromous) were selected as out-groups for genetic comparisons.

The primers (Mäkinen & Merilä 2008) used to amplify the mitochondrial control region fragment were: Forward Primer: (5' CCTTTAGTCCTATAATGGATG) and Reverse: (5' CCGTAGCCCATTAGAAAGAA). PCR was performed using 20 μl reaction volumes consisting of 1x PCR buffer (200 mM Tris-HCL pH 8.4, 500 mM KCL), 1.5 mM $MgCl_2$, 0.2 mM dNTP (Invitrogen), 0.25 U Taq DNA polymerase (Invitrogen), 5 pmol of each forward and reverse primer and approximately 20 ng of template DNA. PCR products were sent to University of Washington's High-Throughput Genomics Unit at the Department of Genome Sciences, where they were purified (using ExoSap-IT) and sequenced on a capillary sequencer. Sequences were aligned, errors manually edited and contiguous sequences were assembled using Sequencher 4.9 (Gene Codes). Further inspection and file conversion was done using GENEIOUS 5.1 (Biomatters Ltd.).

Genetic Diversity and Differentiation

Arlequin 3.5 (Excoffier *et al.* 2005) was used to calculate standard diversity indices and and measures of genetic differentiation for all populations of both microsatellite and mitochondrial sequence data sets (measures of diversity and differentiation defined in Appendix 1). F_{ST}, which is particularly important as a measure of genetic divergence between pairs of populations, was determined and is emphasized in this study. The analysis of molecular variance (AMOVA) was utilized to assess levels of genetic variation among individuals within sampling sites, variation among sites and variation between species types. To test the effect of species type, an AMOVA (1) was constructed comparing anadromous fish vs. resident freshwater fish from the Jim Creek drainage. A second AMOVA (2) compared resident fish from the Jim Creek drainage vs. other resident populations from the Mat-Su Valley, testing the effect of geographical

location. Exact tests of population differentiation were employed using Genepop 4.0 (Rousset 2008) with the parameters set as follows: number of dememorization steps in the Markov chain set to 1000, batches 100 and iterations per batch to 1000.

Genetic Structure Analysis

A Bayesian analysis of genetic clusters was performed using STRUCTURE 2.2 (Pritchard *et al.* 2000). This Bayesian analysis uses a Markov chain Monte Carlo algorithm to group like genotypes into populations based on individual allele frequencies. An admixed model with correlated allele frequencies was used to explore the number of most likely genetic clusters (K) that existed within the microsatellite data. Five independent simulations of each K=1 to K=10 were performed. Simulations began with a "burn in" period of 25,000 iterations to reduce errors in parameter estimates from starting values, followed by 200,000 iterations to determine final parameter estimates. The most likely estimate of K was determined using two methods descried in Pritchard and Wren (2003) and Evanno *et al.* (2005). The former method describes a qualitative estimate of the real number of genetic clusters by the plateau created at the actual K value when the "log of probability of data" (L(K)) is plotted against each successive value of K (Pritchard & Wren 2003). A more formalized method provided by Evanno *et al.* (2005) uses the ad hoc statistic ΔK to determine the true number of genetic clusters. The true K is revealed by a modal peak when plotting a measure of the rate of change between successive L(K) values. After the most likely number of clusters was determined to be four (see results), the mean proportion of membership (m(q)) of each population to a given cluster was determined from the five simulations in which K=4. All ten populations were assigned to one of four clusters based on their highest percentage membership.

A neighbor-joining tree based on Cavalli-Sforza's and Edwards' chord distance D_C (Cavalli-Sforza & Edwards 1967) was created using all ten sample sites and assembled using PHYLIP 3.69 (Felsenstein 2005). This genetic distance measure was shown to be more accurate than other distance measures that do not consider the stepwise nature of mutation in microsatellites, such as Nei's genetic distance (Nei 1972), and has the highest likelihood of constructing the correct tree topology (Takezaki & Nei 1996). Gene frequencies were bootstrapped over microsatellite loci 100 times using SEQBOOT. The CONSENSE program in PHYLIP was used to build a consensus tree of all populations. DRAWTREE was used to display the consensus tree. A second neighbor-joining tree was constructed using the methods described above from gene frequencies of the four population clusters (see results) revealed by STRUCTURE. Haplotypes from the mitochondrial control region sequences were used to construct individual-level and population-level neighbor-joining trees in NTSYSpc v. 2.11-W (Exeter Software), with Nei's genetic distance (Nei 1972) used to determine tree structure. With the individual-level mtDNA tree revealing two major mitochondrial clades (see results), individuals from each clade were tested for inclusion in two major mtDNA haplotype groups known to occur in Alaskan stickleback, the Trans-North Pacific Clade (TNPC) and the Euro North American Clade (ENAC) (Orti et al. 1994; Johnson & Taylor 2004). The testing involved the restriction digest/electrophoresis procedure outlined in Johnson and Taylor (2004).

RESULTS

Genetic Diversity

Estimates of genetic diversity (Table 3) obtained from the amplification of nine microsatellite loci (Appendix 2) were consistent with expectations based on previous surveys of genetic diversity in threespine stickleback that compared the same measures (see Reusch *et al.* 2001; Raeymaekers *et al.* 2005; Mäkinen *et al.* 2008). The different measures of genetic diversity calculated were generally consistent, so the mean number of alleles is emphasized below. The most diverse populations were the anadromous Rabbit Slough and the Jim Lake populations, averaging 18.9 and 18.2 alleles respectively. The anadromous Mud Lake population was slightly lower with 14.9 alleles. The most genetically diverse resident freshwater population was the Wasilla Lake population with genetic diversity measures comparable or surpassing those of the anadromous Mud Lake sample. Genetic diversity was lower in the resident freshwater samples from Jim Lake and Mud Lake relative to the anadromous samples inhabiting the same lakes. The least diverse population was the Tern Lake resident freshwater population with an average of 5.3 alleles. The overall analysis of diversity showed a reduction in allelic diversity in resident freshwater populations compared to anadromous populations.

Analysis of sequences from the mitochondrial control region revealed 40 haplotypes among the 144 specimens sequenced (Table 4). Congruent with the microsatellite data, the Jim Lake anadromous population was among the most diverse populations sequenced containing ten haplotypes. The Mud Lake resident freshwater population was the least diverse with only three haplotypes, none of which were private to the population (i.e., only found in Mud Lake). Consistent with the microsatellite analysis, Wasilla Lake was the most diverse resident

freshwater population. As a whole, resident freshwater populations were less diverse than anadromous populations.

Genetic Differentiation

The exact test of population differentiation revealed differences in all pairs of populations to be highly significant except Jim Lake anadromous/Rabbit Slough anadromous ($p=0.127$). Pairwise F_{ST} values from the analysis of microsatellite loci also indicate significant genetic differentiation among most samples (Table 5). Based on pairwise F_{ST} values, Tern Lake resident stickleback were the most genetically distinct from other stickleback populations, with an average pairwise F_{ST} of 0.24 (SD±0.030). Two pairs of populations exhibited pairwise F_{ST} values that did not differ significantly from 0; Jim Lake anadromous vs. Rabbit Slough anadromous (Mud Lake anadromous showed slight statistical differences), and Jim Lake resident vs. Mud Lake resident 2010 ($p>0.05$). Little differentiation was detected among all anadromous stickleback populations sampled, with an average F_{ST} of 0.01 (SD±0.011). Also, geographically proximate populations showed little genetic differentiation. Mud Lake, Mud Lake 2003, Gull Lake and Jim Lake resident populations, all located in the Jim Creek system, had an average F_{ST} of 0.01 (SD±0.011) among them. The other two populations from the Mat-Su Valley, Wasilla Lake resident and Little Meadow Creek resident, had a pairwise F_{ST} value of 0.027. Genetic divergence increased when comparing populations from separate drainages (Jim Creek vs. other Mat-Su populations) and when comparing anadromous vs. resident freshwater types. The average pairwise F_{ST} value between anadromous stickleback and resident freshwater populations was 0.10 (SD±0.048). Pairwise F_{ST} values from mitochondrial control region sequences (Table 6) were consistent with the microsatellite data. Virtually no differentiation was detected among

resident freshwater populations within the Jim Creek drainage (F_{ST}~0). Also, no differentiation was detected among the three anadromous populations sampled (F_{ST}~0). The Wasilla Lake resident population was the most divergent from all sampled populations, with an average pairwise F_{ST} of 0.49 (SD±0.14), and maximum divergence occurring between it and the anadromous populations (F_{ST}=0.59-0.60). Again, the strongest differentiation occurred between types (average F_{ST}=0.31; SD±0.21), followed by variation associated with geographic distances among sites.

The analysis of molecular variance (AMOVA) on the microsatellite data set (Table 7) revealed that most of the genetic variation exists within populations (>90%). However, AMOVA 1 (anadromous vs. resident freshwater) indicated that 8.12% of the variation exists between anadromous and resident freshwater types and only 1.00% exists among populations within these groups. In comparison, AMOVA 2 (location) does not show a significant component of variance between drainage groups and 5.05% of the variance among populations within drainage groups. Though significant variation between drainage groups was not detected using the AMOVA, pairwise F_{ST} and tree typologies indicate that significant genetic variation exists among resident freshwater populations occurring in different drainage systems, but increases between anadromous and resident freshwater types. AMOVAs conducted on the mitochondrial control region sequence data showed different results (Table 8). AMOVA 1 (species type) showed that 21.26% of variance in the mitochondrial genome exists between anadromous and resident freshwater populations, with no significant variation (~0%) occurring among populations within these groups and 81.65% of the genetic variation existing within populations. AMOVA 2 (location) showed that nearly 49% of the genetic variation (however,

not significant likely due to small sample sizes) existed between the Jim Creek populations (Mud and Jim Lakes) and the Wasilla Lake population. Approximately 5% of the variance occurred among the populations within these groups and roughly 46% occurred within the populations. Comparisons of both mitochondrial AMOVAs indicate that substantial variance in mitochondrial sequences is present between anadromous and resident freshwater species types but the variance is larger between geographical regions.

Population Structure

Neighbor-joining tree topologies from all ten sampled populations suggest divergence among populations based on both species type and drainage (Figure 5). Jim and Mud Lake freshwater samples collected from the Jim Creek system in 2010 cluster together with a bootstrap value of 97, indicating little genetic divergence between them, which is consistent with pairwise F_{ST} values. The Mud Lake resident sample collected in 2003 and Gull Lake resident sample cluster together with a bootstrap value of 60, but belong to a larger cluster containing all resident samples of the Jim Creek drainage (bootstrap=91). The geographically neighboring Wasilla Lake and Little Meadow Creek samples from the Mat-Su Valley also cluster together, and the most geographically distant resident freshwater population, the Tern sample from the Kenai Peninsula, is separate from the rest of the resident freshwater populations. The three anadromous populations sampled cluster together as well, which is also consistent with the low pairwise F_{st} values between them.

Bayesian cluster analysis of these ten populations is consistent with these results. STRUCTURE revealed four genetic subdivisions (K=4) based on a plateau observed when

plotting the L(K) as K increases (Figure 6) and ΔK (Figure 7). These clusters can be broken down by a combination of species type and location. Based on highest mean proportion of membership (m(q)) (Table 9), all populations can be broken down into anadromous, Jim Creek resident freshwater, Mat-Su Valley resident freshwater, and Kenai resident freshwater. Jim Lake anadromous, Mud Lake anadromous and Rabbit slough anadromous populations reported proportional memberships of 0.83-0.90 to the anadromous cluster. Resident freshwater populations from Mud Lake, Jim Lake and Gull Lake had proportional memberships ranging from 0.79 (2003 Mud Lake resident) to 0.95 (Jim Lake and Mud Lake residents). Little Meadow Creek resident stickleback and Wasilla Lake residents showed membership to the Mat-Su Valley resident cluster (0.80-0.88), while Tern Lake residents posted a proportional membership of 0.97 to the Kenai resident freshwater cluster (this was the only population sampled from the Kenai Peninsula). A neighbor-joining tree based on STRUCTURE-generated allele frequencies (Figure 8) depicts the relationships among the four clusters. Analysis of the estimated membership coefficients for each individual in each cluster (Q) (Figure 9) reveals individuals that do not correspond to their population's inferred cluster. For instance, Wasilla Lake residents have an 88% membership to the Mat-Su cluster, but the other 12% share similarities with both the Jim Creek and anadromous clusters. The Mud Lake 2003 samples showed the smallest proportion of individuals assigned to a cluster, 79% belonging to the Jim Creek cluster. Approximately 10% of the Mud Lake 2003 samples were associated with the Mat-Su Cluster, while the other 10% were assigned to the anadromous cluster. This trend was consistent for all populations; fish associated to genetic clusters other than their population's inferred cluster did not occur more frequently in any one specific cluster. Instead, fish associated to clusters other than their population's inferred cluster were generally split between multiple clusters.

A neighbor-joining tree based on mitochondrial control-region sequences reveals genetic structuring that is consistent with the microsatellite analysis for the Jim Creek drainage populations (Figure 11). Anadromous populations of Rabbit Slough, Jim Lake and Mud Lake appear to be closely related. Mud Lake and Jim Lake resident freshwater populations are also genetically similar, while the Wasilla Lake resident freshwater population is the most divergent. A second neighbor-joining tree comparing all 144 individuals sequenced revealed two major mitochondrial clades (Figure 10), consistent with previous research on mtDNA variation of stickleback in the Pacific Northwest (Orti et al. 1994; Johnson & Taylor 2004). Genetic testing shows that the larger of the two mtDNA clades, consisting of approximately 75% of all specimens sampled, belongs to the ENAC mitochondrial clade. The other 25% belong to the more rare mitochondrial clade known as TNPC (Johnson & Taylor 2004). Surprisingly, approximately 75% of the Wasilla Lake resident population that was sampled belongs to TNPC, compared to approximately 33% from Jim Lake residents, 29% from Mud Lake residents and 5% from anadromous populations. These findings support the highly divergent nature of the mtDNA haplotype frequencies for the Wasilla Lake resident population demonstrated by pairwise F_{ST} values between it (Table 6) and the other populations sampled and the overall structure of the population level tree (Figure 10). Furthermore, this observed high level of TNPC within Wasilla Lake is probably also responsible for the variation detected in the mitochondrial control region between drainage groups (AMOVA 2 -Table 8), which is inconsistent with the low levels of variation between the same groups in the microsatellite data (AMOVA 2 - Table 7).

DISCUSSION

Research on the parallel evolution of threespine stickleback species pairs has contributed greatly to our understanding of the way species evolve under divergent natural selection imposed by environmental heterogeneity (McKinnon & Rundle 2002). First reported in the literature by Karve *et al.* (2007), a morphological assessment revealed the presence of a lake-resident/anadromous stickleback pair within Mud Lake, Alaska breeding in sympatry. Here, we present the first genetic analysis of a sympatric lake-resident/anadromous species pair of threespine stickleback, conducted on the species pair inhabiting Mud Lake and the Jim Creek drainage. The analysis in this study will provide a model for comparison with other stickleback species pairs, which will further add to our understanding of the factors driving adaptive radiation within the threespine stickleback species complex. In addition, the mounting research on the species pair in Mud Lake should facilitate future efforts to study the mechanisms that have contributed to the evolution of reproductive isolation between anadromous and resident freshwater stickleback.

Evidence and Magnitude of Genetic Divergence

Results from the genetic analysis support a previously conducted morphological survey (Karve *et al.* 2007) detecting the presence of a reproductively isolated pair of threespine stickleback within Mud Lake, Alaska. In addition, this study identifies other sites in the Jim Creek drainage where anadromous and resident freshwater stickleback breed sympatrically. Data were collected on the sympatric species pair occurring in Jim Lake, but anadromous stickleback were observed at other sites (e.g., Gull Lake) and likely occur throughout the Jim Creek drainage. The anadromous populations in the Jim Creek drainage were highly divergent

genetically from the resident freshwater stickleback in the drainage, and clustered with another anadromous population sampled from the Mat-Su Valley, not with the resident populations inhabiting the lakes in which they breed. Anadromous populations reported little divergence among them with an average F_{ST} value of 0.01 (SD±0.009), less genetic differentiation than reported by previous studies comparing anadromous populations (F_{ST}=0.018-0.075 in Raeymaekers *et al.* 2005; F_{ST}=0.06 in Mäkinen *et al.* 2008). All the resident freshwater samples from the Jim Creek drainage clustered together. When comparing species type, the average F_{ST} value between anadromous and resident freshwater stickleback samples in the Jim Creek drainage was 0.09 (SD±0.008), which falls under the category of moderate genetic differentiation on a scale established by Wright (Conner & Hartl 2004). Consistent with our findings, Reusch *et al.* (2001) reported average pairwise F_{ST} values between anadromous and resident of 0.14 (SD±0.008), which also falls into the category of moderate differentiation. Similarity, Raeymaekers *et al.* (2005) reported F_{ST} values between European populations of resident and anadromous ranging 0.109 to 0.253. Pairwise F_{ST} values between sympatrically breeding anadromous and resident freshwater stickleback samples in the Jim Creek drainage were greater than those between the two resident stickleback samples in the neighboring Mat-Su Valley, which do not breed sympatrically with anadromous stickleback. The average F_{ST} values between the Little Meadow Creek and anadromous samples was 0.06 (SD±0.003) and the average F_{ST} between the Wasilla Lake sample and the anadromous samples was also 0.06 (SD±0.008). This indicates that the resident freshwater stickleback in the Jim Creek drainage are as divergent genetically, or more divergent, from the anadromous stickleback with which they breed sympatrically (in the same lake), than resident freshwater populations in neighboring drainages that do not breed sympatrically with anadromous fish. Tern Lake residents were more

divergent from anadromous populations than all the other resident populations sampled, with an average F_{ST} value of 0.21 (SD±0.02). The variation described above is also detected in the AMOVA comparing species pair types of the Mud Lake system. Though most of the variation exists among individuals within populations, significant variation exists between species pair types of the Mud Lake system (AMOVA 1 – Table 7).

Within the Jim Creek drainage, the four resident samples examined are genetically very similar, indicating a high degree of gene flow between these populations, likely due to their geographic proximity and the incomplete land barriers separating the lakes. The resident Mud Lake and Jim Lake populations sampled in 2010 had a pairwise F_{ST} value that did not differ significantly from 0, indicating that stickleback in these two lakes interbreed enough that they can be considered a single gene pool. There was not a significant component of variance in allele frequencies segregating between them. Although small, the Gull Lake sample collected in 2010 exhibited statistically significant pairwise F_{ST} values when contrasted with the other samples of resident stickleback collected in the Jim Creek drainage, indicating some significant population genetic structure among resident stickleback within the Jim Creek drainage (F_{ST}=0.02; SD<0.001). This is significant because the geographic distances among sites are small. Although fish can likely swim between all the sites in the drainage sampled, there seems to be enough geographic structuring in the system to significantly constrain the free exchange of alleles, impeding genetic homogenization throughout the system. In addition, as indicated by pairwise F_{ST} values and the structure of the neighbor-joining trees, Mud Lake resident specimens sampled in 2010 show slight divergence from samples taken at the same site in 2003 (F_{ST}=0.02; SD±0.003); however, levels of genetic diversity appear to have remained steady. Genetic drift

can cause allele frequencies to change over time (Frankham *et al.* 2010), and given the small magnitude of the genetic divergence between the 2003 and 2010 samples, random genetic drift likely accounts for the observed difference. However, other processes such as limited gene flow between andromous and resident forms cannot be ruled out.

Anadromous populations were uniform genetically and were typically more diverse than resident populations, consistent with previous empirical findings (Mäkinen *et al.* 2008; Reusch *et al.* 2001). Previous studies employing microsatellites have documented heterozygosity values on the order of 0.72-0.85 for anadromous fish and 0.58-0.73 for resident freshwater and stream populations, as was the general trend in this study (anadromous 0.80-0.88; freshwater 0.52-0.80) (Mäkinen *et al.* 2008; Reusch *et al.* 2001). The higher levels of genetic diversity are likely attributable to the larger sizes of anadromous stickleback populations compared to resident freshwater populations and to resident populations going through genetic bottlenecks when they are established since only a small subset of individuals in any oceanic population will establish a given resident population (Frankham *et al.* 2009). The observed low genetic divergence among anadromous populations most likely can be attributed to a high degree of gene flow among anadromous stickleback breeding in different areas and spending most of their lives in fairly homogeneous oceanic environments (e.g., DeWoody & Avise 2000). However, Mud Lake anadromous fish showed significant genetic differentiation from the other two anadromous populations sampled and exhibited slightly lower genetic diversity. Although genetic diversity was lower in Mud Lake anadromous specimens, it clearly fell into the range of diversity indices characteristic of anadromous fish. For instance, heterzygosity levels for Mud Lake anadromous fish were 0.85, consistent with the range for other anadromous populations (see above). This

genetic divergence between Mud anadromous and Jim anadromous-Rabbit Slough anadromous was unexpected and may be worth examining in future studies. It suggests that there is a slight level of genetic differentiation between the anadromous stickleback in Mud Lake relative to those in neighboring Jim Lake and the anadromous Rabbit Slough population in the Mat-Su Valley, making them at least partially genetically segregated from other anadromous fish. Additionally, it must be noted that our Mud Lake anadromous fish had a higher rate of unsucessfully amplified microsatellite loci (14 fish did not amplify for some loci), possibly due to factors like DNA quality of some speciemens. If the missing loci contained rare alleles common to anadromous fish, it could account for the slight genetic divergence from the other anadromous populations.

Analysis of out-group populations from the Mat-Su Valley and Kenai Peninsula indicates significant genetic differentiation among resident freshwater populations associated with geographical location. Populations in the Jim Creek drainage differed from the other populations of the Mat-Su Valley by an average F_{ST} of 0.06 (SD±0.01). Increasing genetic divergence with increasing geographic distance is a common pattern observed in nature (Frankham *et al.* 2009) and specifically among resident freshwater stickleback populations (e.g., Reusch *et al.* 2001). This detected genetic differentiation is likely due to isolation after initial colonization, followed by genetic drift in which gene frequencies randomly shift over time (Frankham *et al.* 2009). Tree topologies also support the genetic structure described above.

Proportional membership values from the Bayesian genetic cluster analysis clearly support a high degree of reproductive isolation between the anadromous and resident freshwater

populations; anadromous populations represent a distinct cluster separated from resident populations (Table 9). Although high (>78%), genetic membership of individuals to each cluster was not complete (Table 9). For instance, the Little Meadow Creek resident stream population had an 80% membership to the Mat-Su Valley cluster. However, a majority of the remaining individuals from Little Meadow Creek that were not assigned to this cluster fell almost equally between the anadromous and Jim Creek clusters. Similarly, the resident Mud Lake sample taken in 2003 reported 79% of its members belonging to the Jim Creek resident freshwater cluster while the remaining members were associated with both the anadromous and Mat-Su resident clusters. Even Wasilla Lake, which had an 88% membership to its cluster, had the remaining individuals distributed between anadromous and Jim Creek clusters. This was a common theme for individuals assigned to genetic clusters other than their population's inferred cluster. Individuals that were not assigned to their population's inferred cluster rarely associated completely with another single cluster. There could be two reasons for this pattern. 1) A small amount of gene flow exists among all fish sampled, or 2) lineage sorting for these post-glacial populations is not complete, meaning that every gene has not converged to the overall phylogeny of the population. Due to the topography of the region, a lack of evidence for an anadromous-resident intermediate hybrid (Karve *et al.* 2007; Bell *et al.* 2010) and the genetic divergence detected in this study, it is unlikely that gene flow is occurring. If it were occurring in some populations, allele frequencies would likely be biased toward one cluster or another. The most likely explanation is that lineage sorting for these populations is not complete and these specimens contain rare alleles that are also found in other populations. For example, analysis of estimated membership coefficients (Q) reveal approximately 1-2 individuals from the Mud Lake resident 2003 population that are classified as anadromous and 1-2 individuals classified as Mat-

Su fish. Hybridization could be the reason for this but high F_{ST} values relative to Wasilla Lake and Little Meadow Creek residents do not support introgression between anadromous and resident fish in the Jim Creek drainage. Furthermore, the distance and topography between Mud Lake and the other populations from the Mat-Su Valley (Wasilla and Little Meadow Creek) would make it very difficult for these populations to hybridize. The most likely explanation is that a small number of individuals from the Mud Lake 2003 population still contain low frequency alleles or combinations of alleles that were once common in marine ancestors, and share these alleles with anadromous and Mat-Su fish. In contrast, Tern Lake, which is a high elevation lake (approx. 315 m above sea level) located on the Kenai Peninsula, had a very high correct assignment rate (97% membership to its own genetic cluster). This could be because Tern Lake residents may represent an older population compared to the Mat-Su and Jim Creek populations, and lineage sorting is more complete. Alternatively, an anadromous population from the Kenai Peninsula that is genetically different from the ones we sampled in the Mat-Su Valley and Jim Creek drainage could have founded Tern Lake.

Assessment of the mitochondrial diversity among populations was generally consistent with the microsatellite analysis. Anadromous fish were overall genetically homogeneous, with little differentiation among populations. Resident populations from the Jim Creek drainage were genetically very similar, in accordance with the microsatellite data. More diversity was found in anadromous fish than in resident freshwater populations. Pairwise F_{ST} analysis and tree topologies support significant genetic differentiation between anadromous and resident freshwater types. Surprisingly, Wasilla Lake residents were extremely divergent from the five other populations surveyed in the mitochondrial analysis. This divergence was also detected in

the AMOVA comparing populations differing in geographical location, in which more genetic divergence was detected between populations of different local than between anadromous and freshwater fish (Table 8). Further analysis revealed that Wasilla lake residents contained an unusually high percentage of a mitochondrial clade that is typically rare in the Pacific Northwest. Orti *et al.* (1994) identified two ancient mitochondrial lineages within threespine stickleback that differ in approximately 2-3% of sequence. The Euro-North American clade (ENAC) is found throughout northern Europe and North America, including the west coast from northern California to Alaska. The trans-north Pacific clade (TNPC), native to the coastal waters near the seas of Japan, is rare in frequency (only about 6% in marine stickleback) compared to ENAC throughout eastern Pacific waters, which is consistent with our anadromous samples (5% TNPC). A previous survey of the TNPC in freshwater lakes in Alaska revealed a range of 0-25% TNPC (Johnson & Taylor 2004). Our Jim and Mud resident populations showed levels of TNPC of 29% and 33%, respectively, which is comparable to the 25% reported for Mud Lake by Johnson and Taylor (2004). The frequency of TNPC in Wasilla Lake was approximately 75%, which is extremely high. However, certain lakes and streams in the Pacific Northwest have been known to contain high percentages of TNPC, in some instances greater than 90%, with some populations even becoming fixed for the TNPC (i.e., TNPC = 100%) (Deagle *et al.* 1996; Thompson *et al.* 1997; Johnson & Taylor 2004). It is unclear what is responsible for this unusually high frequency of TNPC in freshwater lakes and streams, like Wasilla Lake. Random genetic drift could result in a shift in allele frequencies over time. Starting with an ancestral population that harbors low percentages of TNPC, most resident freshwater populations are expected to retain low percentages of TNPC or become fixed for the more common ENAC (i.e., TNPC = 0%). However, given enough random samples, some populations may drift to high

frequencies of TNPC by chance. Alternatively, selective processes may be acting on a mitochondrial gene, resulting in a higher fitness payoff in certain freshwater environments. What the particular gene or selective mechanism would be is not known. Although scoring of the TNPC vs. ENAC clades is typically done using the mitochondrial *Cytochrome b* gene (Orti et al. 1994, Johnson and Taylor 2004), mitochondrial genes are linked because the mitochondrial genome does not recombine. Therefore, natural selection, if acting on mitochondrial haplotypes, could be acting on any of the mitochondrial genes. Whatever the cause, understanding why certain resident freshwater lake populations exhibit high frequencies of TNPC merits further attention.

Addressing Pre and Post-Zygotic Selection

Genetic anaylsis revealed significant genetic divergence between anadromous and resident stickleback breeding sympatrically in the Jim Creek drainage, with little evidence of significant genetic introgression. Since the means for maintaining genetic isolaion are unclear, several possible mechanisms based on previous stickleback research are outlined below.

The lack of obvious hybridization does not eliminate the possibility of low levels of hybridization that is undectable in moderate genetic surveys like this one. Lab experiments have shown that allopatric resident freshwater males will occasionally breed with anadromous females carrying large clutches of eggs (Furin 2006). However, hybridization may be rare enough that hybrids do not contribute significantly to the gene pool, and this could be exacerbated if hybrids have lower fitness (see below). Despite the possibility of hybridization, the species pair in Mud and Jim Lakes are maintaining separate gene pools. Other pre-zygotic factors may be playing a

large role in this process. Previous studies on an anadromous/river-resident population of stickleback have shown that spatial and temporal isolation may contribute to the lack of hybridization (Hagen 1967). Karve *et al.* (2007) showed that the peak breeding times for anadromous and resident fish may differ by as much as five weeks, with resident fish peaking in male nuptual coloration before anadromous fish. Furthermore, differential catch rates of species type per location indicated that anadromous and resident stickleback within Mud Lake may exhibit spacial preferences when nesting. However, these temporal and spatial differences in breeding preferences are only partial; there is broad overlap between resident and anadromous stickleback regarding when and where they breed in Mud Lake, allowing the potential for hybridization to remain significant. Positive assortative mating due to morpholgical differences may also be a factor contributing to the genetic isolation observed in this study. Assortative mating between benthic and limnetic stickleback ecotypes (Nagel & Schluter 1995) and between resident freshwater and anadromous stickleback ecotypes is well known (Hay & McPhail 1975, McKinnon *et al.* 2004, Furin 2006). Body size has been shown to contribute to assortative mating in stickleback (Nagel & Schluter 1998; McKinnon et al. 2004; Boughman et al. 2005) and may provide a basis for the assortative mating between the species pairs in the Jim Creek drainage. Anadromous fish are substantially larger than resident lake fish in this system. For instance, Karve *et al*. (2007) reported the standard length of Mud Lake residents to be 49.4 mm (SD±4.8) compared to 69.2 mm (SD±3.2) in Mud Lake anadromous fish. In addition, assortative mating would likely be an advantage for the two types because the relative fitness of hybrids compared to parents would likely be less. In benthic/limnetic pairs of stickleback, hybrids have lower fitness due to the high degree of ecological differences within habitats (Schluter 1995). With lake-anadromous hybrids, the biggest impediment for increased hybrid survival is likely the

life history switch from andromous to resident due to major differences in migratory behavior and physiological processes (Johannesson 2001). We cannot be certain to what extent each of these pre or post-mating factors are responsible for maintaining reproductive isolation. Future studies concetrating on isolating mechanisms between anadromous and resident Mud Lake stickleback comprise a major direction for future research. Although genetic divergence between anadromous and resident forms was significant, the resident population is postglacial and has evolved from anadromous stickleback, probably like those presently breeding in the Jim Creek drainage, within the last 15,000 years. Thus understanding how reproductive isolation is maintained between these closely related but morphologically and ecologically divergent forms can help us to better understand the mechanisms at play during the early stages of speciation.

Evolution of Reproductive Isolation

The timeframe in which reproductive isolation within Mud and Jim Lake resident freshwater stickleback occurred is unclear. Our current knowledge of the Matanuska river drainage suggests that lakes in this area likely formed within the last 9,500-15,000 years (Reger & Pinney 1996); although evolution to a resident form may have occurred on a more contemporary time scale. Bell *et al.* (2004) showed that a nearly monomorphic population of a complete lateral plate morph of recently established resident freshwater stickleback in neighboring Loberg Lake, Alaska evolved into a population consisting mostly of low lateral plate morph fish within 12 years (1990-2001). This rapid adaptation to freshwater lakes and streams, and the corresponding morphological evolution, may be made possible by major alleles such as *Eda*, which is responsible for low-plate morphology in stickleback (Colosimo *et al.* 2005) and the more recently discovered *Pitx1* enhancer, associated with pelvic reduction (Chan *et al.* 2010).

The Mud Lake resident freshwater sample collected in 2003 was quite similar genetically to the resident freshwater samples collected in the system in 2010 but still very distinct from the anadromous samples, indicating that the resident population in Mud Lake has likely been established and reproductively isolated from anadromous stickleback in the system for some time. This is also supported by the level of genetic divergence at microsatellite loci and of the mtDNA control region. Pairwise F_{ST} values between resident and anadromous stickleback samples in the Jim Creek drainage were greater than those between resident freshwater populations in the neighboring Mat-Su Valley and anadromous populations. Since anadromous stickleback do not occur where the resident freshwater populations in the Mat-Su Valley were sampled, this suggests that the resident form has been established in the Jim Creek drainage as long as resident freshwater populations in neighboring drainages. That is, the resident stickleback populations in the Jim Creek drainage are not newly derived resident freshwater populations. Whether the mechanisms of reproductive isolation developed in allopatry or sympatry, the members of this species pair are behaving like distinct biological species, though the potential of interbreeding still remains.

Conclusion

The first genetic assessment of a lake-resident/anadromous species pair of threespine stickleback is presented in this study. Results from this study support our four predictions about the genetic diversity and differentiation between the species pair. 1) Based on neutral genomic markers and mitochondrial DNA sequences, significant genetic differences exist between anadromous fish and resident fish in the Jim Creek drainage. Genetic diversity indices clearly show genetic differences between anadromous fish and resident freshwater fish which are

consistent with previous findings (see above). Furthermore, tree typologies and measures of genetic differentiation support genetic structure between anadromous fish and resident fish of the Jim Creek drainage. 2) Bayesian cluster analysis and the neighbor-joining trees revealed that resident fish in the Jim Creek drainage genetically cluster together, representing a group distinct from anadromous fish. 3) Although complete reproductive isolation cannot be confirmed, this study finds little evidence that resident freshwater fish from the Jim Creek drainage are hybridizing with anadromous fish in sympatry. This is supported by the existence of separate genetic clusters for lake-resident and anadromous stickleback in the Jim Creek drainage. 4) More convincingly, we support our prediction for a lack of hybridization by showing the magnitude of genetic divergence between andromous and resident samples in the Jim Creek drainage is similar to the magnitude of divergence between anadromous fish and other resident fish living in allopatry. Studies on the mechanisms that maintain reproductive isolation between these two forms represent a major direction of future research.

Secondary findings from this study include the documentation of anadromous fish throughout the Jim Creek drainage. We also found small amounts of genetic structure among resident populations of the Jim Creek drainage. Futhermore, we found statistically supported divergence between Mud Lake populations collected in 2003 and the same population collected in 2010. Lastly, we found a high frequency of individuals from an ancient mitochondrial clade, TNPC, within Wasilla Lake. Whether this phenomenon has occurred by random genetic drift or selection is unclear. Investigation of the frequency of the TNPC clade should be done on other lakes in the region. Additionally, more work needs to be done analyzing sequence variation of the mitochondrial genome. With current DNA sequencing rates, surveying the entire

mitochondrial genome of several threespine stickleback individuals could reasonably be achieved. This could provide insight into whether natural selection is acting on mitochondrial haplotypes, contributing to the high levels of TNPC found in some lakes.

This study provides a baseline for future research. As we have previously established, threespine stickleback species pairs have proven to be of great value to researchers that seek to answer questions about how morphological structures evolve and how new species form. Given the magnitude of morphological and ecological divergence between these sympatric forms and the lack of apparent hybridization between them, the species pair in the Mud Lake system can help answer these questions. As a genetically and mophologically defined species pair of threespine stickleback, this pair should be enticing to researchers wanting further insight into the phenotypic and genotypic changes that allow new species to evolve and the mechanisms that maintain reproductive isolation.

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TABLES
AND
FIGURES

Table 1. Summary of Sampled Alaskan Threespine Stickleback Populations. N is the number sampled from each site. Latitudes and longitudes are approximate locations of trapping sites.

Location	Species Type	Population Abbreviation	Year Collected	N	Region	Coordinates
Jim Lake	Anadromous	JimA	2010	48	Jim Creek	61 33 26.3 N, 148 55 25.8 W
Mud Lake	Anadromous	MudA	2010	48	Jim Creek	61 33 52.6 N, 148 56 45.6 W
Rabbit Slough	Anadromous	RS	2010	48	Mat-Su Valley	61 32 30.2 N, 149 13 56.5 W
Jim Lake	Resident Freshwater	JimR	2010	48	Jim Creek	61 33 26.3 N, 148 55 25.8 W
Mud Lake	Resident Freshwater	Mud	2010	48	Jim Creek	61 33 52.6 N, 148 56 45.6 W
Mud Lake	Resident Freshwater	MudR03	2003	48	Jim Creek	61 33 52.6 N, 148 56 45.6 W
Gull Lake	Resident Freshwater	Gull	2010	48	Jim Creek	61 32 29.1 N, 148 57 19.2 W
Tern Lake	Resident Freshwater	Tern	2010	48	Kenai Peninsula	60 32 03.3 N, 149 32 56.7 W
Wasilla Lake	Resident Freshwater	Was	2010	48	Mat-Su Valley	61 34 53.4 N, 149 25 39.6 W
Little Meadow Creek	Stream	MdCrk	2010	48	Mat-Su Valley	61 34 09.1 N, 149 45 36.3 W

Table 2. Details of the 9 microsatellite loci used to examine genetic differentiation of Alaskan threespine stickleback populations. LG, linkage group; MP, multiplex panel.

Loci	LG	MP	Size-Range	Fluorescent Dye
*Stn110	IX	I	134-223	FAM
**4170PBBE	III	I	100-199	HEX
Stn33	III	I	127-194	NED
Stn171	XV	II	104-176	FAM
Stn195	XX	II	114-191	HEX
Stn67	VI	II	160-250	NED
7033PBBE	XI	III	159-258	FAM
Stn120	X	III	144-180	HEX
Stn168	XIV	III	148-199	NED

*Stn loci described in Peichel *et al.* (2001).
**PBBE loci described in Largiader *et al.* (1999)

Table 3. Standard diversity indices from microsatellite loci used to examine genetic differentiation in Alaskan threespine stickleback populations. N, number of specimens; mA, mean number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity.

Population	N	mA	H_O	H_E
JimA	48	18.2	0.822	0.871
JimR	48	11.8	0.692	0.756
MdCrk	48	13.6	0.677	0.801
MudA	48	14.9	0.686	0.846
MudR	48	11.9	0.632	0.745
MudR03	48	12.1	0.674	0.758
Gull	48	13.9	0.719	0.773
RS	48	18.9	0.792	0.883
Tern	48	5.33	0.407	0.519
Was	48	14.6	0.757	0.809

Table 4. Standard diversity indices from mitochondrial control region sequence data of Alaskan threespine stickleback populations. N, number of specimens; H, number of haplotypes; N_e, effective number of alleles; HD, haplotype diversity; %PA, percent of private alleles.

Population	N	H	n_e	HD	%PA
JimA	24	10	4.2456	0.8009	70
JimR	24	5	2.3226	0.5942	40
MudA	24	7	3.3611	0.7359	28.6
MudR	24	3	2.2677	0.5833	0
RS	24	7	3.2061	0.7194	28.6
Was	24	8	2.4202	0.6123	50

Table 5. Pairwise F_{ST} values generated from microsatellite data to examine genetic differentiation of Alaskan threespine stickleback populations.

	JimA	JimR	MdCrk	MudA	MudR	MudR03	MudRSwp	RS	Tern	Was
JimA	~									
JimR	0.09750**	~								
MdCrk	0.06574**	0.05908**	~							
MudA	0.01687**	0.08970**	0.06010**	~						
MudR	0.10367**	0.00298	0.06099**	0.09035**	~					
MudR03	0.08027**	0.01332**	0.04589**	0.08233**	0.01801**	~				
MudRSwp	0.08935**	0.01510**	0.05684**	0.09191**	0.01505**	0.00487**	~			
RS	0.00120	0.09180**	0.06225**	0.01606**	0.09634**	0.07543**	0.08461**	~		
Tern	0.20374**	0.26495**	0.24462**	0.22648**	0.26578**	0.26714**	0.25201**	0.18978**	~	
Was	0.05871**	0.06698**	0.02657**	0.07260**	0.07319**	0.04401**	0.05511**	0.05888**	0.24464**	~

*$p<0.05$
**Remains significant after Bonferroni correction

Table 6. Pairwise F_{ST} values generated from mitochondrial control region sequence data to examine genetic differentiation of Alaskan threespine stickleback populations. Negative F_{ST} values are approximately zero.

	JimA	JimR	MudA	MudR	RS	Was
JimA	~					
JimR	0.18977**	~				
MudA	-0.03937	0.18716	~			
MudR	0.13949	-0.03722	0.13584	~		
RS	-0.02924	0.20579**	-0.03333	0.15230**	~	
Was	0.59276**	0.32115**	0.59449**	0.35062**	0.60680**	~

*$p<0.05$
**Remains significant after Bonferroni correction

Table 7. Analysis of molecular variance AMOVA for microsatellite data. d.f., degrees freedom; % variation, percentage of variation accounted for by each factor; VC, variance component; P, p values.

Effect	d.f.	% variation	VC	P
Amova 1				
Mud/Gull/Jim Resident v. Anadromous (species type)	1	8.12	0.081	0.030
Among populations within groups	5	1.00	0.011	<0.001
Within populations	581	90.88	0.091	<0.001
Amova 2				
Mud/Gull/Jim v. Was/MdCrk (geographical location)	1	1.41	0.014	0.176
Among populations within groups	6	5.05	0.051	<0.001
Within populations	654	93.55	0.065	<0.001

Table 8. Analysis of molecular variance AMOVA for mitochondrial control region sequence data. d.f., degrees freedom; % variation, percentage of variation accounted for by each factor; VC, variance component; P, p values.

Effect	d.f.	% variation	VC	P
Amova 1				
Mud/Gull/Jim Resident v. Anadromous (species type)	1	21.26	0.213	0.099
Among populations within groups	3	-2.91	-0.037	0.989
Within populations	110	81.65	0.183	0.003
Amova 2				
Mud/Jim v. Was (geographical location)	1	48.8	0.488	0.180
Among populations within groups	4	5.06	0.099	<0.001
Within populations	133	46.15	0.539	<0.001

Table 9. STRUCTURE designated populations. N, number of specimens; m(q), mean proportion membership

Population	N	Type	Location	Inferred Cluster	Abbreviation	m(q)
JimA	48	Anadromous	Matanuska Drainage	Anadromous	Anad	0.90
MudA	48	Anadromous	Matanuska Drainage	Anadromous	Anad	0.83
RS	48	Anadromous	Matanuska Drainage	Anadromous	Anad	0.90
JimR	48	Resident Freshwater	Matanuska Drainage	Jim Creek	JimC	0.95
MudR	48	Resident Freshwater	Matanuska Drainage	Jim Creek	JimC	0.95
MudR03	48	Resident Freshwater	Matanuska Drainage	Jim Creek	JimC	0.79
Gull	48	Resident Freshwater	Matanuska Drainage	Jim Creek	JimC	0.90
MdCrk	48	Resident Stream	Susitna Drainage	Mat-Su Resident	MatSu	0.80
Was	48	Resident Freshwater	Susitna Drainage	Mat-Su Resident	MatSu	0.88
Tern	48	Resident Freshwater	Kenai Penninsula	Kenai Resident	Kenai	0.97

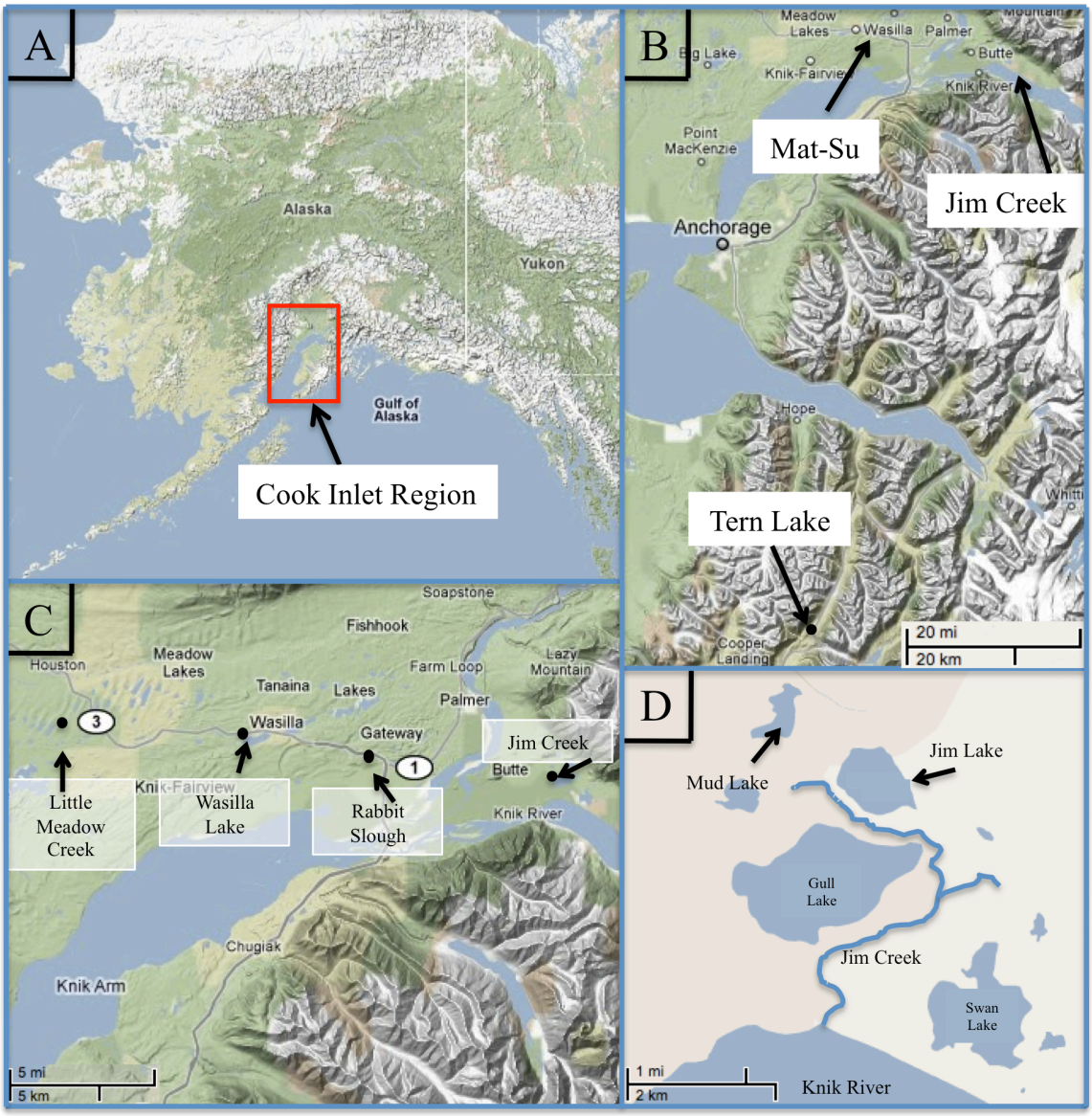

Figure 1. A) Image of Alaska indicating Cook Inlet region. B) Cook Inlet region indicating location of Mat-Su Valley, Jim Creek drainage and Tern Lake. C) Zoomed image of Mat-Su Valley. D) Image of Mud Lake system in the Jim Creek drainage.

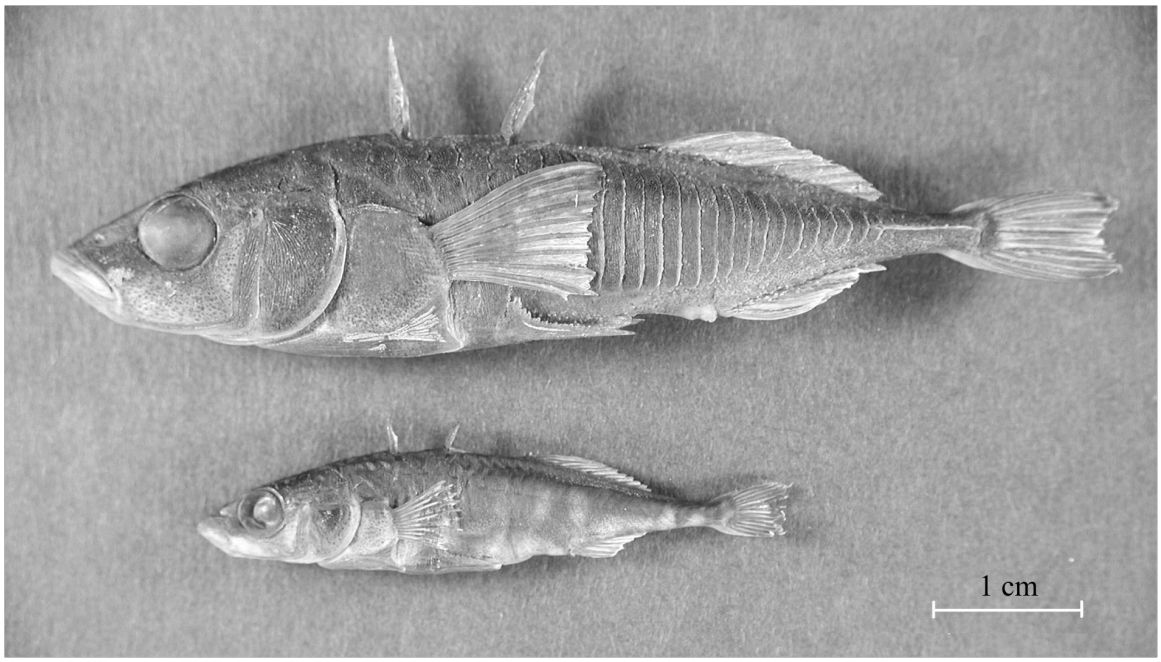

Figure 2. Species pair of threespine sticklback in Mud Lake, Alaska. Top: Anadromous type stickleback; Bottom: Resident freswater stickleback (Karve *et al.* 2007).

Figure 3. Geographical distribution map of the threespine stickleback. Shaded regions represent areas inhabited by the threespine stickleback (Bell & Foster 1994).

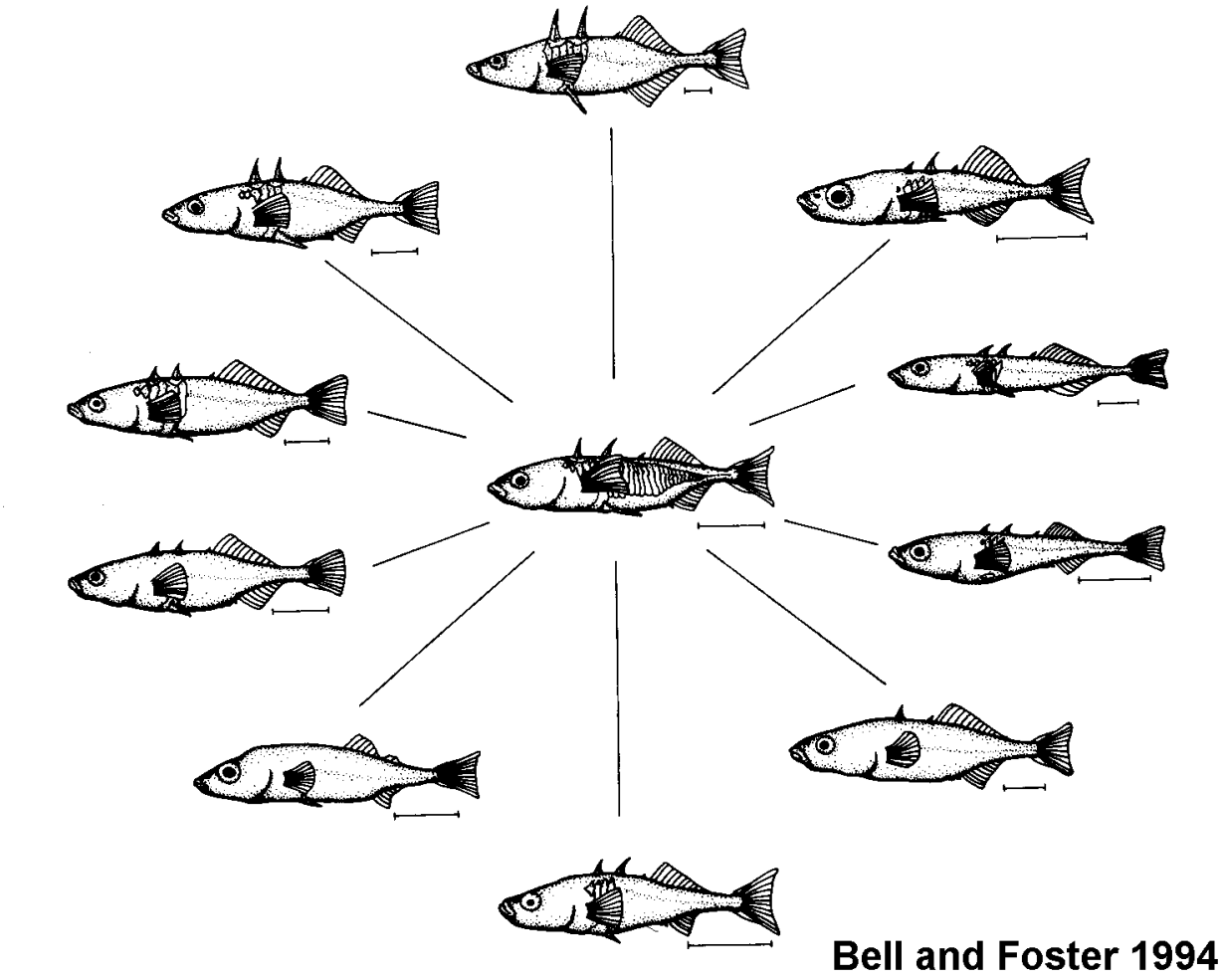

Figure 4. Image depicting adaptive radiation of threespine stickleback that can occur when marine fish are introduced into freshwater. Middle fish is marine type; fish on outer wheel are sketches of resident freshwater populations (Bell & Foster 1994).

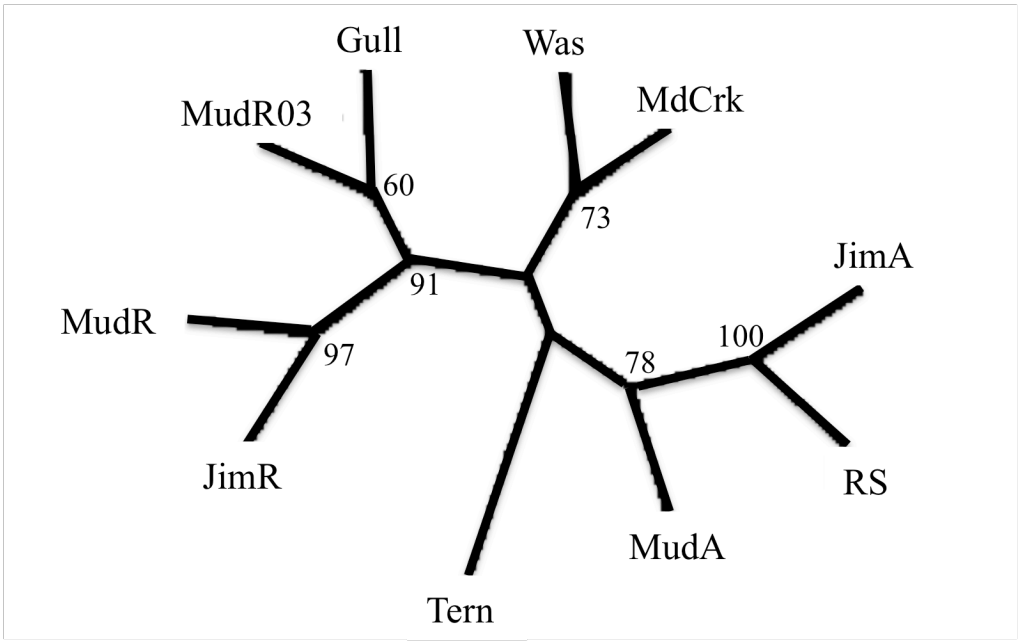

Figure 5. Neighbor-joining tree for specified sampling areas a priori populations based on microsatellite data. Bootstrap values are from 100 replicates with values >50% presented. Population abbreviations listed in Table 1.

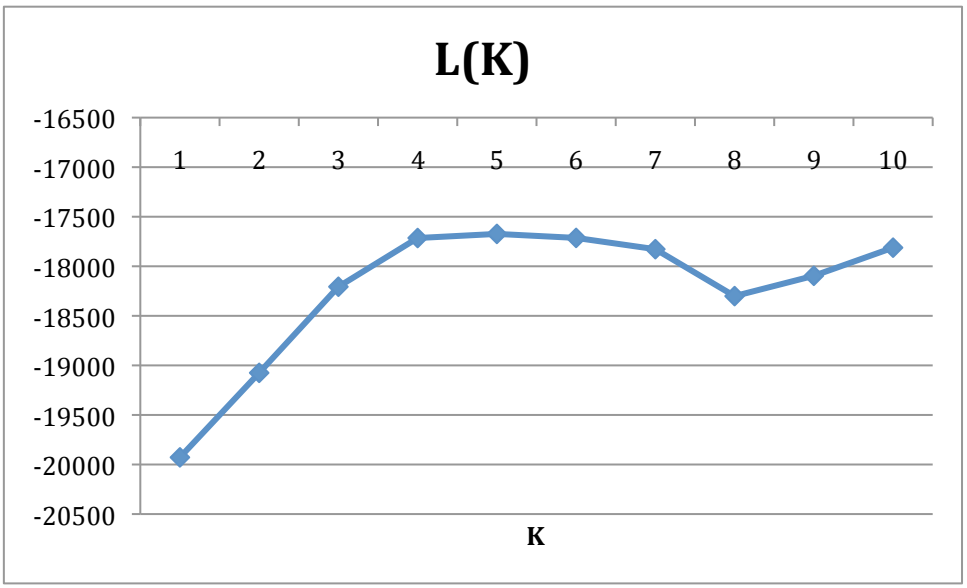

Figure 6. Graph used to help determine the actual number of genetic clusters (K) as determined by STRUCTURE. Pritchard *et al.* (2000) indicates the true value of K as defined when the plateau of the "log of probability of data" (L(K)) has been reached as K increases, indicated here as K=4.

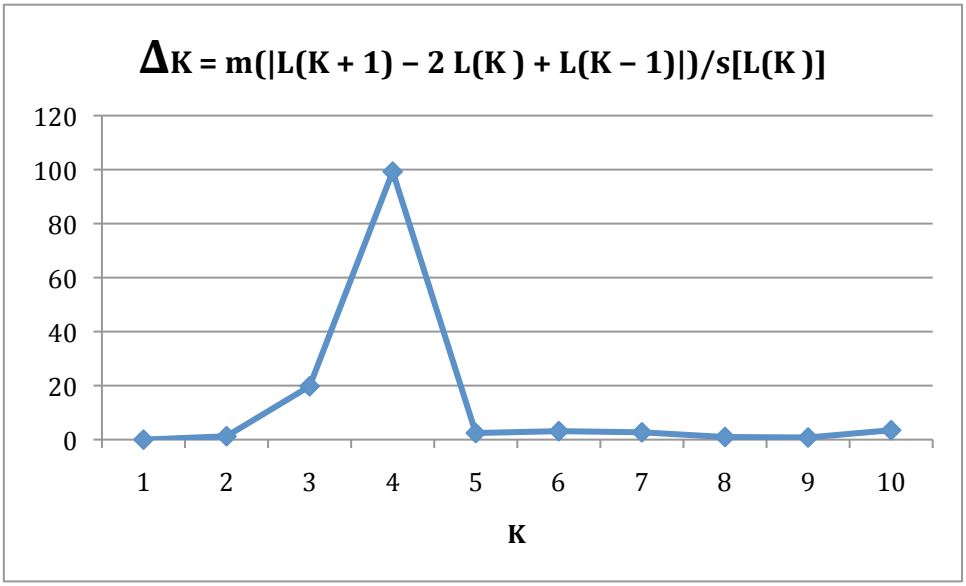

Figure 7. Graph used to help determine the actual number of genetic clusters (K) as determined by STRUCTURE. Evanno *et al.* (2005) defines the actual value of K by the modal displayed when graphing an algorithm based on the ΔK as K increases, indicated here as K=4.

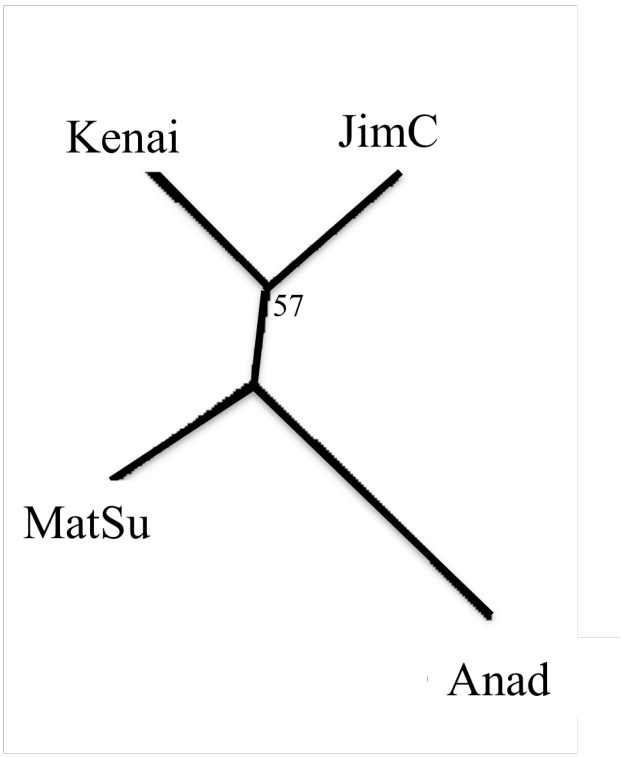

Figure 8. Neighbor-joining tree for STRUCTURE designated populations based on microsatellite data. Bootstrap values are from 100 replicates with values >50% presented. Cluster abbreviations and a priori population membership to each cluster listed in Table 9.

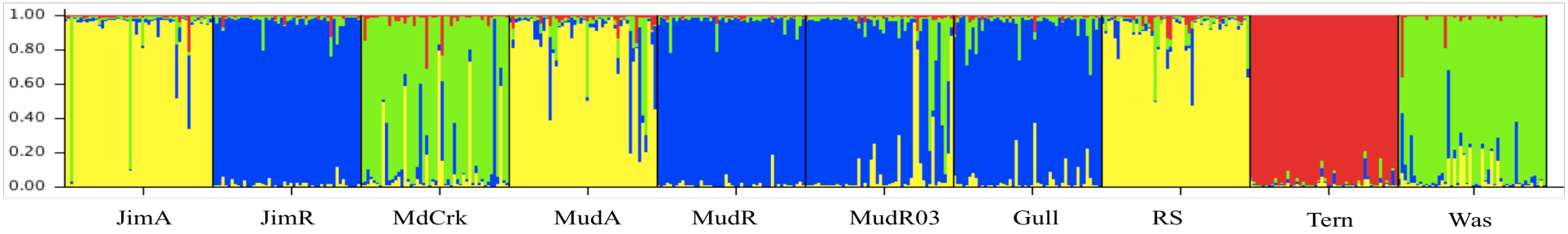

Figure 9: Summary plot of the estimated membership coefficients for each individual, in each cluster (Q). Each individual is represented by a single vertical line broken into K (K=4) colored segments, with lengths proportional to each of the K inferred clusters. The population abbreviations are located in Table 1.

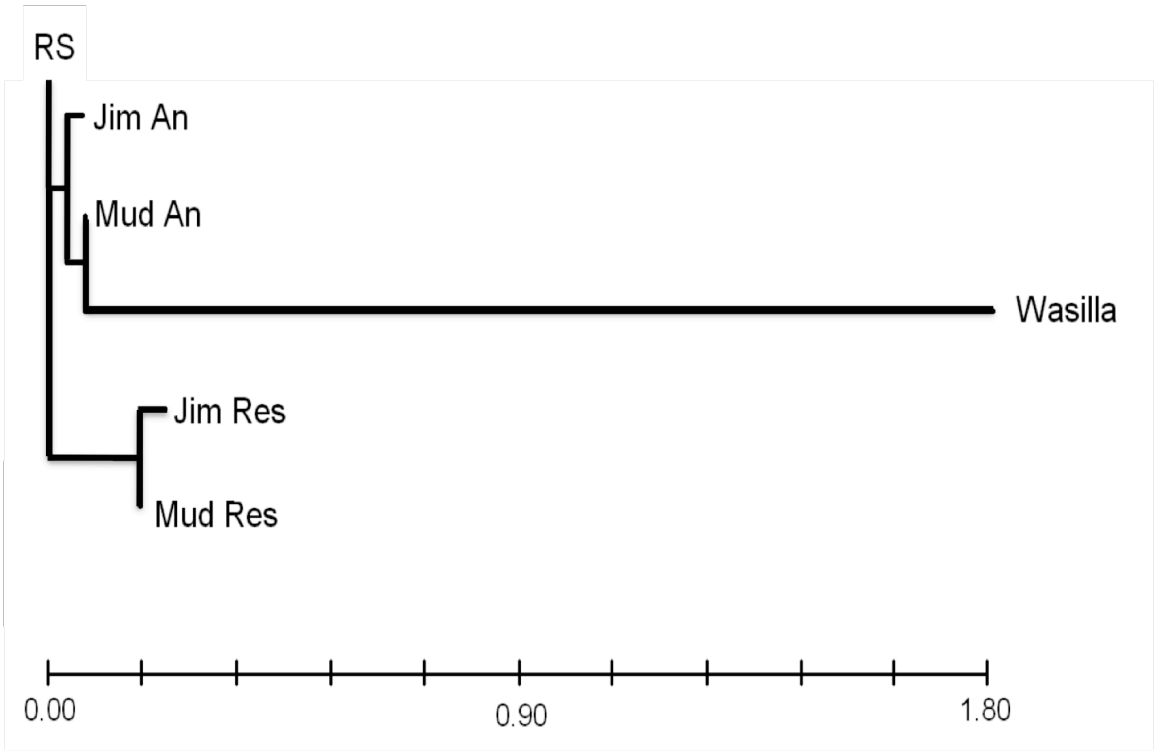

Figure 10. Neighbor-joining tree for specified sampling areas based on mitochondrial sequence data. Node assignments calculated using Nei's genetic distance measure (Nei 1972).

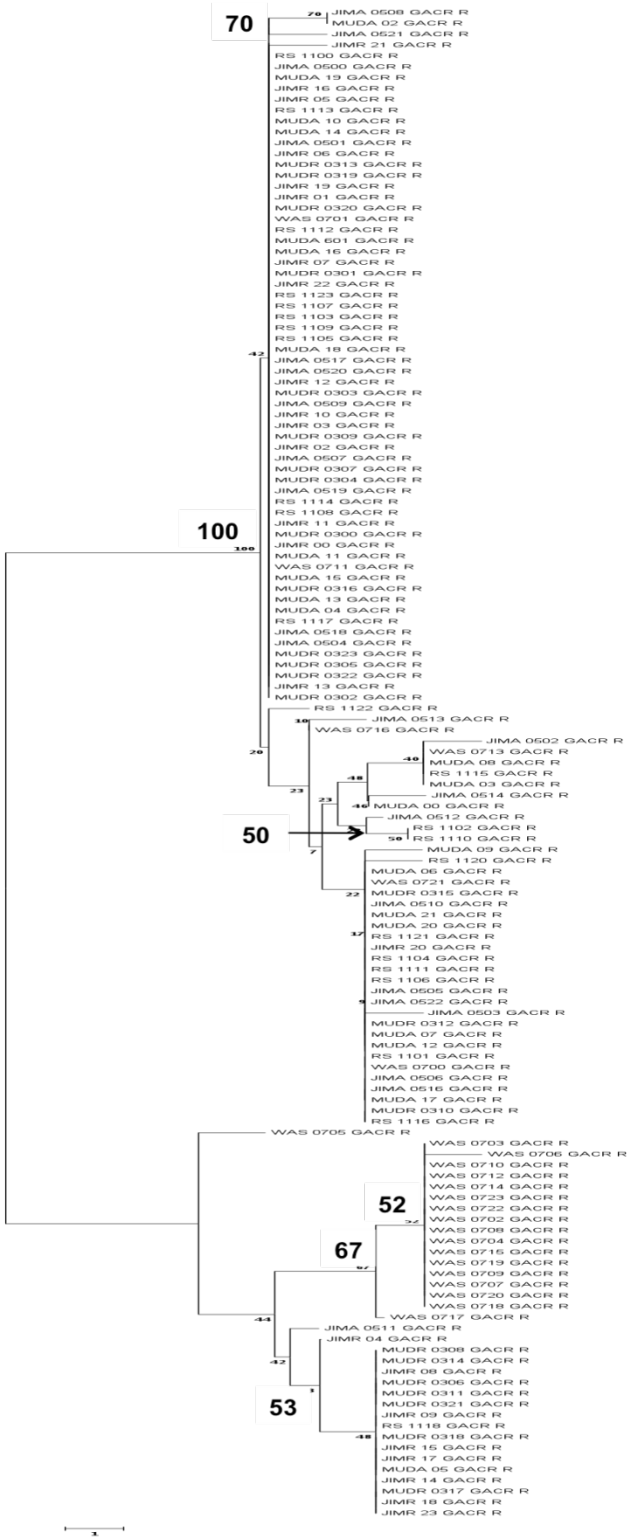

Figure 11. Neighbor-joining tree for all individuals based on based on mitochondrial sequence data. Bootstrap values are from 100 replicates with values >50% presented. Upper clade (larger) identified as ENAC; Lower clade (smaller) identified as TNPC (Johnson and Taylor 2004).

Appendix 1. Definitions of measures of genetic diversity and differentiation used in this study.

AMOVA	Different hierarchical Analysis of Molecular Variation to evaluate the amount of population structure (Excoffier *et al.* 2005)
Exact Test of Genetic Differentiation	Test of non-random distribution of haplotypes into population samples under the hypothesis of panmixia (Excoffier *et al.* 2005).
F statistics	Measures of total inbreeding in a population (F_{IT}), partitioned into that due to inbreeding within sub-populations (F_{IS}) and that due to differntiation among sub populations (F_{ST}) (Frankham *et al.* 2009)**.** Following Wright, F_{ST} values from 0.05 to 0.15 are considered to indicate moderate genetic differentiation, between 0.15 and 0.25 indicate great differentiation, and greater than 0.25 indicate very great differentiation (Conner and Hartl 2004).
Effective Number of Alleles (n_e)	The number of alleles that if equally frequent would result in the observed heterozygosity (Frankham *et al.* 2009).
Expected Heterozygosity (H_E)	The heterozygosity expected for a random mating population with the given allele frequencies according to the Hardy-Weinberg equilibrium (Frankham *et al.* 2009).
Haplotype Diversity (H)	A measure of genetic diversity among haplotypes (Frankham *et al.* 2009).
Heterozygosity	Proportion of heterozygous individuals for a locus in a population (Frankham *et al.* 2009).
Genetic Distance	A measure of the genetic difference between allele frequencies in two populations or species, e.g. Nei's genetic distance (Frankham *et al.* 2009).

Appendix 2. Table of microsatellite alleles. Question marks indicate unknown alleles.

	STN110		G4170		STN33		STN171		STN195		STN67		GAC703		STN120		STN168	
pop = JimA																		
JimA_01	171	173	108	117	134	136	119	125	175	175	181	192	185	185	168	180	152	167
JimA_02	171	190	111	113	140	143	119	123	175	175	202	226	185	201	168	172	161	167
JimA_03	169	194	108	115	136	140	119	119	166	179	162	169	213	231	150	161	155	161
JimA_04	200	217	117	132	143	145	119	119	160	166	164	169	185	225	150	150	164	176
JimA_05	162	192	150	152	134	143	119	119	166	172	169	169	185	233	153	163	161	165
JimA_06	192	192	113	117	136	136	119	125	179	179	169	179	185	195	157	170	161	176
JimA_07	171	196	154	154	140	143	113	119	170	176	171	177	185	185	150	150	153	175
JimA_08	188	217	108	144	134	136	119	125	166	172	162	181	195	195	153	153	165	165
JimA_09	179	204	111	111	134	140	113	119	166	179	162	171	185	227	150	153	149	176
JimA_10	168	171	105	105	134	136	119	123	166	175	175	222	193	197	153	172	167	176
JimA_11	190	203	111	192	136	136	119	127	175	175	175	179	185	199	150	164	170	197
JimA_12	171	196	108	192	140	143	113	119	166	178	175	177	195	251	153	168	172	182
JimA_13	169	194	108	109	140	150	112	123	175	175	175	186	185	247	150	168	162	164
JimA_14	169	176	144	144	132	145	112	117	175	179	183	186	201	201	150	153	154	172
JimA_15	169	171	113	150	134	145	113	119	170	174	177	177	185	197	150	168	153	176
JimA_16	173	190	104	109	134	140	112	125	166	179	183	188	185	185	150	150	176	176
JimA_17	188	192	108	113	136	186	113	113	178	178	169	169	201	241	150	168	171	175
JimA_18	166	188	108	108	140	140	119	119	160	165	164	188	185	185	153	168	167	172
JimA_19	169	173	125	150	134	136	110	123	172	172	167	183	197	249	150	163	171	182
JimA_20	162	194	108	148	143	192	113	119	170	179	171	179	185	241	150	172	165	167
JimA_21	168	169	147	150	134	143	113	119	166	179	169	175	195	241	164	168	162	170
JimA_22	169	173	112	150	136	140	117	119	179	179	177	190	195	235	161	163	160	176
JimA_23	169	171	111	116	134	143	119	123	166	166	173	196	185	185	150	150	164	175
JimA_24	176	188	108	111	134	134	110	112	166	176	179	181	195	227	161	168	153	176
JimA_25	183	194	113	192	136	194	123	125	166	175	167	179	185	197	152	168	165	167
JimA_26	194	213	108	108	136	140	119	125	175	179	179	188	193	195	150	170	170	172
JimA_27	171	192	111	119	136	140	112	119	166	175	169	181	185	195	150	161	152	162
JimA_28	180	191	147	192	134	140	119	125	172	178	169	214	185	195	150	153	172	174
JimA_29	169	213	109	115	134	136	112	119	175	185	169	181	185	258	168	178	167	175

JimA_30	196	215	113	113	136	140	112	123	175	178	181	186	?	?	?	?	?	?					
JimA_31	166	192	105	150	134	134	119	119	175	178	173	179	191	197	150	161	160	162					
JimA_32	168	173	116	116	136	140	110	123	166	179	175	179	185	185	150	153	162	171					
JimA_33	169	169	104	116	136	143	119	123	178	180	167	177	185	195	150	150	154	167					
JimA_34	173	185	111	111	143	143	?	?	174	174	164	164	185	239	150	152	152	165					
JimA_35	169	171	111	112	136	136	119	123	178	182	162	179	164	185	164	164	149	165					
JimA_36	166	197	109	109	134	145	113	119	175	178	169	169	185	193	172	178	174	177					
JimA_37	180	186	112	112	134	136	119	119	166	176	162	162	197	197	152	152	161	165					
JimA_38	168	171	107	144	134	134	116	119	170	175	179	205	195	199	168	172	155	161					
JimA_39	183	213	111	150	140	143	116	125	180	180	162	186	185	185	168	168	165	192					
JimA_40	169	190	108	111	140	143	116	119	167	170	175	229	185	195	153	172	149	176					
JimA_41	173	188	109	109	140	140	112	113	167	182	179	183	195	195	152	168	160	175					
JimA_42	169	174	108	192	134	136	119	123	166	178	177	190	185	191	153	153	164	165					
JimA_43	196	219	108	115	136	136	117	119	166	175	173	235	191	195	153	164	164	179					
JimA_44	169	188	108	111	140	148	123	123	167	167	186	196	185	191	150	168	148	171					
JimA_45	169	219	108	109	145	148	119	125	174	179	162	179	193	195	150	164	175	180					
JimA_46	173	173	108	113	134	140	113	119	170	172	162	175	185	201	153	153	165	176					
JimA_47	177	196	108	150	134	136	112	119	165	179	169	224	227	237	150	153	165	165					
JimA_48	186	217	115	144	140	148	113	129	174	180	162	190	197	201	150	153	161	162					
pop = JimR																							
JimR_01	169	176	105	150	139	150	119	122	166	172	175	198	213	213	168	170	162	177					
JimR_02	169	185	112	112	134	136	119	125	178	183	173	186	213	213	150	152	154	162					
JimR_03	173	196	109	111	134	134	112	125	170	182	175	186	213	213	152	152	160	160					
JimR_04	171	188	111	150	134	143	113	125	170	182	162	186	213	213	150	170	165	177					
JimR_05	188	192	109	111	134	134	119	136	178	180	179	179	213	213	150	153	167	177					
JimR_06	176	185	108	110	134	155	119	125	176	178	162	188	213	213	152	170	160	162					
JimR_07	?	?	?	?	?	?	119	125	160	171	?	?	?	?	152	168	160	160					
JimR_08	169	188	108	108	134	134	119	123	172	176	173	175	213	213	150	153	161	161					
JimR_09	182	192	110	116	134	137	125	125	?	?	?	?	213	213	152	152	162	175					
JimR_10	171	192	109	116	134	155	125	136	179	183	167	205	213	213	153	168	161	175					
JimR_11	168	217	109	150	134	134	119	136	172	172	179	179	213	213	167	168	164	167					
JimR_12	169	191	105	111	140	155	125	129	170	176	186	209	213	213	152	152	160	162					
JimR_13	171	192	108	108	134	134	129	136	167	182	179	188	213	213	150	152	161	176					

JimR_14	?		?		?		?		?		119	125		166	182	179	231	213	213	153	153	154	162
JimR_15		171		192		110		117		140		155	125	125		180	180	173	214	213	213	150	153	164	175
JimR_16		169		192		112		195		134		150	119	125		167	180	186	188	213	213	153	153	164	164
JimR_17		171		171		112		116		136		137	119	119		175	182	186	186	213	213	153	153	161	176
JimR_18		173		191		112		112		145		194	113	125		160	178	186	211	213	213	152	152	160	162
JimR_19		191		191		108		113		134		165	119	125		179	179	179	186	197	213	152	152	161	177
JimR_20		171		192		109		111		134		134	119	119		167	179	179	198	213	213	152	152	154	162
JimR_21		171		192		111		117		134		150	113	136		176	182	162	179	213	213	150	161	154	162
JimR_22		169		185		112		193		134		155	119	119		179	179	162	198	213	213	153	170	155	164
JimR_23		180		192		109		112		134		134	119	125		166	182	179	186	213	233	152	152	162	177
JimR_24		169		188		109		119		134		155	113	113		178	179	186	240	213	213	150	153	160	166
JimR_25	?		?		?		?		?		?		119	119		170	183	162	198	213	213	153	168	154	155
JimR_26	?		?		?		?		?		?		119	119		182	182	188	198	213	213	168	168	153	155
JimR_27		169		185		110		110		134		134	125	125		179	179	171	205	213	213	153	153	154	161
JimR_28	?		?		?		?		?		?		113	125		171	183	175	211	197	213	153	168	175	177
JimR_29	?		?		?		?		?		?		119	125		170	182	175	183	213	213	170	170	160	166
JimR_30	?		?		?		?		?		?		119	127		166	172	186	202	213	213	153	153	161	162
JimR_31	?		?		110		116		139		140		119	125		171	179	175	179	195	213	153	153	154	162
JimR_32		185		192		111		115		137		137	119	125		178	179	179	198	213	213	150	153	154	160
JimR_33		169		185		109		110		134		139	119	119		175	182	162	175	213	213	150	153	161	175
JimR_34		168		171		109		116		137		150	112	125		176	178	175	179	213	213	153	168	161	161
JimR_35		191		217		109		110		134		134	113	113		182	182	173	186	213	213	153	170	161	177
JimR_36		169		180		108		109		134		148	110	125		171	179	173	175	213	213	153	153	162	168
JimR_37		169		185		111		111		134		134	119	125		175	182	186	211	213	213	150	153	164	168
JimR_38		180		192		109		116		134		136	113	125		175	179	175	188	197	213	153	153	154	162
JimR_39		169		186		107		109		134		134	113	119		182	182	183	188	213	213	153	153	164	164
JimR_40		180		217		110		112		134		150	112	119	?		?	169	169	213	213	153	153	161	162
JimR_41		168		217		111		150		136		136	110	125		179	179	205	205	213	213	150	153	162	175
JimR_42		192		192		112		152		134		150	112	125		178	179	169	202	213	213	150	152	164	177
JimR_43		188		188		108		111		134		136	119	125		175	182	202	205	195	213	168	168	164	177
JimR_44		168		171		108		119		134		155	110	125		176	176	186	186	213	213	153	168	155	161
JimR_45		185		191		110		117		134		134	119	125		182	182	171	179	213	213	152	168	154	162
JimR_46		171		176		109		111		134		139	119	125		172	175	175	188	213	213	168	168	161	166

JimR_47	171	192	109	109	139	194	119	119	167	167	173	214	213	213	153	153	161	164
JimR_48	171	188	111	111	134	134	119	125	166	172	181	188	195	213	153	153	164	168
pop = MdCrk																		
MdCrk_01	180	194	108	193	136	140	119	125	179	179	179	186	?	?	?	?	?	?
MdCrk_02	169	221	108	109	137	140	125	125	183	183	188	196	195	199	153	153	164	184
MdCrk_03	171	174	108	152	140	140	119	125	176	176	179	211	213	213	153	153	165	176
MdCrk_04	180	188	107	147	140	145	119	125	176	176	171	205	199	209	153	153	165	176
MdCrk_05	169	171	133	193	137	140	110	125	166	166	179	179	209	213	153	153	157	178
MdCrk_06	169	169	152	193	137	140	?	?	?	?	?	?	195	199	150	172	178	178
MdCrk_07	179	186	193	193	136	143	119	125	179	179	173	173	213	213	150	153	178	182
MdCrk_08	197	219	154	193	137	143	125	125	172	180	202	202	203	213	150	150	174	179
MdCrk_09	188	221	108	193	137	140	125	125	178	179	162	167	213	213	152	152	162	177
MdCrk_10	174	179	100	107	137	140	113	125	167	176	179	179	213	213	150	152	177	182
MdCrk_11	169	188	108	148	136	137	125	125	126	179	186	205	199	213	153	153	177	177
MdCrk_12	?	?	?	?	?	?	125	125	170	178	179	183	199	209	150	153	177	177
MdCrk_13	171	179	192	192	136	140	113	125	180	183	188	211	195	213	150	150	154	179
MdCrk_14	171	186	108	112	137	140	119	125	182	182	183	205	213	213	150	153	175	177
MdCrk_15	169	200	108	108	136	145	113	119	166	178	179	226	213	213	150	155	162	165
MdCrk_16	171	190	108	197	136	137	125	125	176	178	181	202	195	213	161	161	178	178
MdCrk_17	179	194	107	107	137	137	119	119	175	179	179	186	199	213	153	161	155	164
MdCrk_18	169	171	107	108	127	143	?	?	?	?	?	?	203	203	150	152	176	177
MdCrk_19	188	194	150	192	137	137	?	?	?	?	?	?	213	221	152	152	165	177
MdCrk_20	?	?	?	?	?	?	?	?	?	?	?	?	213	213	153	161	154	176
MdCrk_21	169	194	107	150	136	140	?	?	?	?	?	?	213	213	153	163	179	188
MdCrk_22	183	191	108	108	134	140	?	?	?	?	?	?	195	213	150	153	165	190
MdCrk_23	169	194	108	108	127	140	?	?	?	?	?	?	213	213	153	153	178	178
MdCrk_24	173	194	108	108	140	143	?	?	?	?	?	?	213	213	150	153	177	182
MdCrk_25	169	194	107	192	137	140	119	129	175	178	175	186	213	213	150	161	177	177
MdCrk_26	190	190	144	144	136	136	119	125	178	178	190	209	205	213	150	153	160	179
MdCrk_27	169	173	110	199	134	143	119	125	175	175	179	205	195	213	150	152	154	177
MdCrk_28	171	215	107	192	140	140	119	119	179	183	211	224	213	213	161	161	175	182
MdCrk_29	169	194	107	107	137	140	125	136	179	183	175	214	213	213	163	172	155	162
MdCrk_30	169	179	107	108	136	137	?	?	?	?	175	175	203	213	150	150	177	190

MdCrk_31	169	190	111	112	140	143	119	125	179	179	171	205	213	213	152	152	162	165
MdCrk_32	169	194	152	152	137	143	119	119	167	172	179	183	213	213	150	153	155	177
MdCrk_33	183	188	107	150	136	137	119	119	175	179	175	198	209	215	150	150	178	179
MdCrk_34	194	200	107	107	143	145	119	119	166	179	190	196	203	213	150	153	165	165
MdCrk_35	169	171	108	193	136	136	119	119	176	178	175	179	201	209	153	153	154	178
MdCrk_36	190	190	109	109	136	140	119	125	176	183	162	169	201	213	150	153	166	176
MdCrk_37	169	174	107	107	137	140	119	125	178	178	186	188	199	215	150	174	154	178
MdCrk_38	185	185	193	193	140	140	113	119	170	182	175	188	199	213	153	164	177	182
MdCrk_39	183	183	108	112	134	140	119	119	175	178	173	200	213	213	153	153	155	178
MdCrk_40	169	171	193	196	137	143	119	119	180	180	179	179	203	213	153	153	167	170
MdCrk_41	180	194	107	147	136	140	119	125	179	183	179	211	203	203	150	153	177	177
MdCrk_42	171	188	107	107	137	140	113	119	170	175	173	179	213	213	150	174	177	177
MdCrk_43	185	221	107	192	136	137	119	119	175	175	175	179	213	213	153	153	162	177
MdCrk_44	185	196	111	111	134	137	119	125	179	182	179	211	213	213	150	153	154	177
MdCrk_45	197	221	111	147	136	137	125	125	175	175	175	209	203	213	153	153	175	177
MdCrk_46	191	200	111	147	136	143	117	119	171	171	162	175	195	199	150	152	177	177
MdCrk_47	194	194	193	193	134	140	119	119	166	166	179	186	213	213	150	150	165	177
MdCrk_48	169	169	112	112	136	143	119	136	?	?	?	?	199	213	153	153	177	177
pop = MudA																		
MudA_01	177	194	108	109	136	190	119	125	171	171	173	186	185	185	150	152	154	167
MudA_02	?	?	?	?	?	?	112	125	176	176	175	175	195	195	150	153	153	165
MudA_03	?	?	?	?	?	?	125	136	171	179	186	207	193	197	150	168	174	175
MudA_04	166	192	109	192	134	134	119	119	166	178	167	205	195	254	164	170	152	167
MudA_05	162	188	108	111	136	148	122	125	178	179	186	229	185	237	150	161	148	174
MudA_06	164	164	129	154	132	134	125	125	178	178	179	188	195	221	150	153	162	192
MudA_07	190	223	108	109	134	140	119	119	166	183	175	186	185	193	153	168	149	161
MudA_08	173	191	116	117	136	136	?	?	?	?	?	?	185	185	150	164	167	176
MudA_09	?	?	?	?	?	?	119	125	172	172	167	211	185	199	150	150	160	162
MudA_10	169	191	112	113	134	134	119	125	172	172	179	181	195	258	150	150	160	175
MudA_11	171	171	108	108	134	143	119	125	178	179	162	186	185	195	153	153	154	175
MudA_12	169	173	111	111	134	134	125	125	180	180	175	186	185	191	153	159	162	167
MudA_13	177	185	107	144	134	157	119	119	178	180	177	205	185	185	150	150	167	167
MudA_14	185	217	109	192	136	145	113	119	183	183	186	198	195	195	153	153	161	161

MudA_15	186	194	111	150	136	140	119	125	167	172	196	205	185	185	153	168	177	177
MudA_16	185	190	107	107	150	190	119	119	180	185	175	205	195	195	150	150	164	178
MudA_17	171	192	108	148	136	140	?	?	?	?	?	?	195	195	150	150	165	176
MudA_18	?	?	?	?	?	?	113	125	172	183	179	186	185	195	153	168	167	176
MudA_19	168	169	109	109	140	145	?	?	?	?	?	?	185	199	153	172	167	175
MudA_20	185	185	108	108	132	134	113	136	172	172	175	179	193	193	150	153	165	165
MudA_21	217	217	109	109	136	136	113	125	167	167	181	186	185	191	153	168	162	176
MudA_22	196	200	109	112	136	137	?	?	?	?	?	?	193	227	150	157	160	162
MudA_23	169	185	108	108	136	148	?	?	?	?	?	?	227	237	150	150	165	165
MudA_24	177	185	108	110	134	136	?	?	?	?	?	?	185	195	168	168	154	165
MudA_25	173	194	108	111	134	143	119	119	172	180	173	179	185	197	150	150	164	167
MudA_26	169	174	107	112	145	148	119	125	166	183	179	186	195	195	153	153	176	179
MudA_27	194	199	107	107	134	140	119	125	179	185	186	205	191	193	153	168	160	192
MudA_28	?	?	?	?	?	?	113	113	179	183	181	186	193	227	150	153	161	165
MudA_29	169	191	108	111	140	140	113	125	180	180	186	205	185	185	150	150	160	160
MudA_30	204	204	109	116	136	165	125	125	185	185	175	186	185	201	153	153	167	172
MudA_31	168	171	109	195	134	134	119	119	166	166	175	202	193	227	153	153	162	167
MudA_32	171	194	120	122	136	140	119	129	167	185	194	202	185	227	153	153	164	176
MudA_33	168	194	109	112	136	140	119	125	172	176	162	186	185	195	168	168	153	179
MudA_34	171	173	113	113	140	143	119	125	185	185	207	207	197	199	168	172	167	174
MudA_35	?	?	?	?	?	?	119	119	176	180	179	179	185	185	150	150	154	170
MudA_36	171	183	108	108	143	143	119	122	172	176	186	209	195	195	153	168	161	164
MudA_37	169	196	107	111	134	145	119	125	?	?	?	?	185	221	168	168	161	165
MudA_38	190	197	119	119	136	140	119	136	176	176	169	179	191	195	150	176	167	174
MudA_39	169	177	110	152	134	145	119	119	176	180	162	175	185	185	150	153	161	176
MudA_40	?	?	?	?	?	?	119	125	162	162	171	200	?	?	153	170	?	?
MudA_41	169	169	112	113	136	137	129	131	180	180	175	205	185	185	153	172	155	176
MudA_42	?	?	?	?	?	?	?	?	?	?	?	?	195	195	150	150	165	174
MudA_43	?	?	?	?	?	?	125	129	167	167	175	186	?	?	150	150	?	?
MudA_44	171	194	108	110	132	140	119	125	172	179	186	186	195	195	152	174	177	177
MudA_45	169	196	107	107	134	134	119	125	183	183	162	205	197	243	150	150	178	178
MudA_46	166	203	111	111	140	143	125	125	171	180	207	214	195	195	150	150	153	164
MudA_47	169	192	105	109	134	140	?	?	?	?	?	?	195	195	150	153	161	172

MudA_48	192	196	105	105	134	134	?	?	?	?	?	?	195	195	153	168	161	164
pop = MudR																		
MudR_01	169	169	109	116	134	134	125	125	179	179	211	218	213	213	150	153	161	161
MudR_02	169	191	105	108	134	137	119	125	171	178	173	186	213	213	170	170	155	161
MudR_03	169	196	111	111	134	139	113	119	171	179	202	202	213	213	153	170	162	177
MudR_04	176	191	111	150	136	150	113	125	175	175	188	188	213	213	150	150	154	161
MudR_05	176	192	107	138	134	137	119	119	171	171	175	196	213	213	153	153	155	178
MudR_06	176	217	192	192	134	137	125	129	171	183	175	179	213	213	168	168	155	176
MudR_07	185	194	108	109	137	155	136	136	178	178	173	211	213	213	153	153	161	164
MudR_08	185	191	109	150	134	137	119	125	179	182	179	205	197	197	170	170	161	170
MudR_09	171	180	111	111	165	194	119	127	183	183	175	179	195	233	153	168	161	164
MudR_10	171	188	107	111	134	148	119	125	166	183	173	175	213	233	152	152	154	161
MudR_11	171	191	108	109	134	134	119	125	179	179	175	198	213	213	150	153	162	165
MudR_12	168	171	109	111	134	145	119	125	179	179	188	188	213	213	153	153	161	164
MudR_13	192	192	111	115	137	150	112	119	175	182	175	186	213	213	150	153	154	162
MudR_14	171	171	108	111	139	150	119	125	179	182	205	205	213	213	153	153	161	164
MudR_15	168	171	116	192	134	137	119	125	180	180	179	250	213	213	168	168	155	177
MudR_16	168	171	109	111	134	134	119	125	?	?	181	186	213	213	153	153	166	170
MudR_17	169	185	109	111	134	134	123	136	182	182	186	198	213	213	152	152	161	165
MudR_18	188	191	110	193	134	134	113	125	179	183	209	209	213	213	150	153	160	199
MudR_19	171	191	111	111	134	155	125	125	166	171	183	205	197	213	152	152	161	162
MudR_20	169	169	108	111	134	139	113	125	175	183	175	175	213	213	153	153	155	177
MudR_21	171	191	107	109	134	150	125	125	183	183	175	179	213	213	153	168	176	177
MudR_22	188	217	108	111	134	155	119	136	171	182	179	179	213	213	150	170	160	177
MudR_23	169	191	105	111	134	155	119	125	160	183	179	205	213	237	152	152	152	162
MudR_24	171	192	110	110	134	139	125	125	160	182	167	186	195	213	153	153	170	176
MudR_25	171	188	108	108	134	139	119	125	166	179	179	209	213	213	152	152	162	167
MudR_26	191	191	111	150	134	155	125	125	171	179	162	186	213	213	150	152	160	160
MudR_27	174	191	107	111	150	155	125	129	175	182	171	179	213	213	150	168	161	176
MudR_28	176	196	111	111	134	145	113	119	166	166	183	205	213	213	152	152	154	160
MudR_29	176	191	109	109	134	140	113	125	182	182	179	214	213	213	153	153	162	162
MudR_30	174	191	110	193	134	137	122	125	180	180	179	205	213	213	153	153	161	164
MudR_31	168	176	111	111	134	134	119	125	175	182	171	188	213	213	153	153	161	164

MudR_32	188	192	108	119	134	136	?	?	?	?	?	?	213	213	150	153	155	161
MudR_33	169	169	108	108	134	134	125	136	178	178	186	214	213	213	161	170	161	162
MudR_34	?	?	?	?	?	?	119	125	172	178	162	188	213	213	150	150	160	160
MudR_35	171	185	109	111	134	155	119	125	166	179	181	211	213	213	153	153	155	177
MudR_36	169	191	108	111	134	134	125	125	172	179	175	175	213	213	153	153	160	162
MudR_37	188	192	111	111	134	134	112	119	172	178	198	205	213	213	152	152	162	176
MudR_38	171	185	108	109	134	137	119	125	172	172	186	186	195	213	150	150	160	167
MudR_39	188	192	111	192	134	134	119	125	183	183	167	211	213	213	153	153	161	175
MudR_40	169	188	108	109	134	134	112	125	178	178	186	186	213	213	153	153	165	175
MudR_41	191	191	108	111	134	134	112	125	170	183	175	179	213	213	152	152	162	167
MudR_42	171	188	111	192	136	155	125	125	175	182	179	218	213	213	150	153	175	175
MudR_43	171	191	108	109	137	155	119	119	179	179	167	198	213	233	150	153	155	175
MudR_44	185	191	109	109	134	134	125	131	178	178	179	186	213	233	150	152	154	160
MudR_45	168	185	108	111	134	134	119	125	178	178	179	179	213	213	150	152	162	177
MudR_46	197	197	108	192	134	137	119	119	160	182	196	205	213	213	153	170	162	177
MudR_47	188	192	111	111	140	155	119	119	166	179	179	207	213	213	153	170	155	161
MudR_48	171	171	109	109	134	134	125	125	170	176	181	196	213	213	152	167	154	165
pop = MudR03																		
MudR03_01	185	191	108	112	134	134	113	127	171	176	173	179	213	213	150	153	154	177
MudR03_02	171	191	115	150	137	194	?	?	?	?	?	?	213	213	152	152	154	165
MudR03_03	171	191	107	108	134	137	117	119	172	176	162	186	213	213	153	168	154	162
MudR03_04	188	191	111	111	134	136	119	119	174	178	171	181	213	213	153	170	155	161
MudR03_05	169	192	111	147	140	155	119	123	176	176	186	186	213	213	144	152	160	161
MudR03_06	171	191	111	115	134	150	119	119	172	175	175	194	213	213	168	168	162	177
MudR03_07	188	192	108	109	134	137	116	119	167	176	173	179	213	213	153	153	162	162
MudR03_08	176	192	108	110	134	150	?	?	?	?	?	?	213	213	153	161	161	164
MudR03_09	176	180	105	116	134	137	117	119	?	?	173	194	213	213	150	150	160	162
MudR03_10	176	192	108	111	134	140	?	?	?	?	?	?	213	213	153	168	162	162
MudR03_11	173	191	111	192	137	137	117	119	167	171	171	192	213	213	144	153	154	165
MudR03_12	169	191	108	109	134	137	119	125	?	?	175	175	213	213	150	168	161	175
MudR03_13	185	191	109	111	137	155	119	125	182	182	175	209	213	213	152	161	162	162
MudR03_14	171	185	109	111	134	136	123	123	176	176	162	175	213	213	152	152	162	162
MudR03_15	169	192	110	193	134	134	119	119	171	176	192	202	213	213	150	150	161	161

MudR03_16	169	188	108	111	134	134	119	125	170	179	167	173	213	213	153	153	164	176
MudR03_17	171	188	108	111	150	155	119	119	164	175	169	198	213	213	150	153	162	175
MudR03_18	185	191	107	147	134	148	112	113	175	175	164	205	213	213	150	153	161	175
MudR03_19	171	191	105	111	134	134	?	?	?	?	?	?	213	213	152	170	160	160
MudR03_20	174	188	107	131	134	134	119	125	176	176	169	186	195	213	152	152	162	177
MudR03_21	171	191	109	192	134	134	119	122	166	179	169	179	213	213	170	170	155	162
MudR03_22	162	191	108	111	134	134	119	119	166	179	181	181	?	?	?	?	?	?
MudR03_23	171	173	107	111	134	140	123	123	170	178	175	179	213	213	150	153	164	165
MudR03_24	171	188	105	111	137	150	119	129	162	162	175	188	213	213	168	168	164	182
MudR03_25	188	191	107	111	134	140	119	119	178	185	173	186	213	213	153	153	155	176
MudR03_26	169	180	112	112	134	140	125	125	170	175	183	194	213	213	152	152	154	160
MudR03_27	171	174	111	119	134	155	119	119	166	175	167	205	213	213	152	170	160	162
MudR03_28	191	194	109	109	134	137	119	119	175	178	186	188	213	213	150	170	155	161
MudR03_29	169	188	108	109	134	155	119	125	179	180	167	177	?	?	?	?	?	?
MudR03_30	171	185	120	193	134	155	119	123	175	179	173	181	213	213	150	152	154	162
MudR03_31	180	204	108	111	136	137	110	113	160	172	169	177	213	213	150	153	161	161
MudR03_32	174	191	109	110	134	137	119	119	167	171	171	183	213	213	153	153	155	161
MudR03_33	171	180	107	111	134	136	117	125	170	170	169	181	213	213	153	153	161	161
MudR03_34	169	169	109	150	134	140	112	125	167	176	171	175	213	213	153	153	155	161
MudR03_35	169	191	107	109	134	136	116	119	162	170	167	175	213	213	168	168	160	175
MudR03_36	171	171	150	150	136	148	110	119	176	176	175	175	185	233	150	170	165	176
MudR03_37	?	?	?	?	?	?	113	119	175	178	171	242	?	?	153	153	?	?
MudR03_38	?	?	108	108	134	134	117	125	167	172	175	186	?	?	153	153	?	?
MudR03_39	192	217	109	110	150	155	119	129	172	178	179	183	213	233	150	168	153	176
MudR03_40	168	171	109	109	134	150	?	?	?	?	?	?	213	213	153	153	161	161
MudR03_41	179	188	111	111	136	136	119	119	?	?	169	169	213	213	153	153	155	161
MudR03_42	169	169	?	?	136	136	119	123	?	?	?	?	?	?	153	153	?	?
MudR03_43	169	169	107	107	134	137	119	119	?	?	?	?	213	213	152	170	164	176
MudR03_44	169	169	109	192	155	165	119	125	176	180	169	173	213	213	150	150	161	167
MudR03_45	171	171	107	112	134	137	119	119	167	180	177	200	?	?	153	153	?	?
MudR03_46	179	179	107	115	134	137	112	119	175	179	173	175	?	?	153	153	?	?
MudR03_47	?	?	?	?	?	?	119	125	166	178	177	211	?	?	?	?	?	?
MudR03_48	?	?	?	?	?	?	116	116	?	?	?	?	?	?	145	153	?	?

pop = Gull																		
Gull_01	180	185	111	119	137	150	113	119	162	176	177	177	195	195	153	153	161	164
Gull_02	191	217	107	111	145	165	119	125	175	175	169	181	213	213	152	152	162	165
Gull_03	134	188	105	192	134	134	119	129	166	170	171	177	213	213	152	161	154	162
Gull_04	192	192	111	111	134	155	112	129	166	170	171	188	213	213	150	170	162	164
Gull_05	169	188	111	115	134	150	119	122	178	190	177	200	213	213	152	152	162	176
Gull_06	169	169	109	116	134	143	119	119	166	179	186	233	213	213	153	153	162	175
Gull_07	169	177	109	109	134	140	112	125	175	175	171	173	213	213	150	153	161	164
Gull_08	169	217	108	111	140	145	112	129	168	176	179	211	213	213	150	153	176	176
Gull_09	174	185	109	111	134	137	112	125	178	178	175	175	213	213	153	153	161	162
Gull_10	169	185	108	108	134	150	119	125	175	175	177	216	213	213	150	152	154	162
Gull_11	180	217	109	111	134	137	119	129	178	179	177	202	213	213	152	168	161	162
Gull_12	171	188	108	111	134	137	119	119	176	179	179	179	197	213	152	168	160	162
Gull_13	169	191	108	111	134	150	119	125	166	175	179	244	213	213	153	153	155	161
Gull_14	176	185	107	111	137	194	119	125	172	175	171	171	197	213	152	152	160	175
Gull_15	168	185	105	111	134	137	119	119	170	175	169	231	213	213	152	152	154	161
Gull_16	174	191	109	192	134	155	112	119	180	180	160	190	213	213	150	153	164	166
Gull_17	176	188	107	109	134	134	119	123	175	175	169	175	213	213	167	170	176	177
Gull_18	188	188	109	110	134	134	129	129	167	180	171	216	213	213	150	153	161	176
Gull_19	171	171	115	115	134	155	125	125	166	178	183	192	197	213	153	153	165	175
Gull_20	174	191	107	109	134	134	119	129	170	179	181	181	213	213	150	153	162	177
Gull_21	171	177	107	150	134	134	119	131	166	178	186	226	213	213	150	153	154	160
Gull_22	171	192	111	192	134	137	104	125	167	179	160	181	213	213	150	153	176	177
Gull_23	168	188	111	192	137	150	119	125	176	176	181	202	213	213	152	152	161	164
Gull_24	174	185	109	192	134	150	112	119	167	182	177	220	213	213	153	153	161	177
Gull_25	176	185	107	111	145	194	112	125	176	179	183	194	213	213	168	170	155	177
Gull_26	191	191	107	111	145	155	119	119	160	170	181	196	213	213	153	153	154	162
Gull_27	171	217	111	116	140	150	119	119	175	179	171	183	195	195	161	168	160	177
Gull_28	171	188	107	192	134	137	112	113	179	182	164	177	213	213	168	168	161	177
Gull_29	171	217	111	192	134	150	119	123	174	175	194	202	213	213	153	153	154	168
Gull_30	168	188	109	116	134	194	119	122	171	176	177	181	213	213	153	153	155	164
Gull_31	191	191	108	109	134	134	125	129	160	175	209	235	213	213	152	152	154	154
Gull_32	176	197	105	192	134	148	119	119	167	180	162	177	213	213	150	150	161	176

Gull_33	180	217	108	192	134	134	112	127	191	191	177	190	213	213	170	170	176	176
Gull_34	169	188	111	116	155	165	112	119	167	176	169	211	213	213	153	168	164	178
Gull_35	185	191	107	111	134	134	119	125	176	180	192	192	213	213	152	152	161	176
Gull_36	162	176	109	111	143	194	112	129	176	182	194	198	213	213	153	153	154	155
Gull_37	168	173	109	150	134	137	123	125	166	176	192	214	213	213	150	153	167	176
Gull_38	168	176	111	111	134	134	122	125	179	180	183	233	213	213	168	170	161	161
Gull_39	169	191	107	109	134	155	125	129	167	167	177	179	213	213	153	153	154	175
Gull_40	168	194	111	111	134	134	129	131	176	180	177	179	213	213	153	153	164	168
Gull_41	166	191	111	111	134	137	119	127	175	187	169	205	213	213	150	153	160	175
Gull_42	169	191	108	111	134	137	119	125	170	176	171	175	213	213	150	153	160	162
Gull_43	171	171	109	192	134	134	112	119	176	178	175	181	213	213	150	170	167	176
Gull_44	169	176	108	109	134	140	112	129	166	179	177	179	197	215	150	153	160	162
Gull_45	169	176	109	147	137	140	112	122	166	180	179	190	213	213	153	168	175	177
Gull_46	180	217	111	115	134	137	123	125	166	167	181	194	213	213	150	153	160	162
Gull_47	188	217	108	117	140	194	129	129	167	176	179	181	213	213	153	153	162	178
Gull_48	192	192	108	111	134	137	?	?	?	?	?	?	213	213	153	170	161	168
pop = RS																		
RS_01	185	188	108	109	134	140	119	119	162	170	169	173	185	185	150	168	167	174
RS_02	176	186	108	108	134	145	119	119	172	178	173	186	191	199	153	153	176	182
RS_03	168	185	150	152	134	136	?	?	?	?	?	?	197	197	150	153	165	165
RS_04	168	196	108	184	134	134	119	119	166	185	231	250	227	241	150	161	164	166
RS_05	173	173	110	110	134	134	119	119	160	179	183	250	195	195	150	170	161	165
RS_06	162	173	115	116	134	155	116	119	170	170	173	175	185	197	150	153	177	177
RS_07	171	194	148	148	134	136	123	127	166	175	229	238	185	197	150	153	153	165
RS_08	169	188	119	192	134	136	113	119	172	179	167	167	191	195	150	172	161	172
RS_09	169	169	111	117	134	148	125	127	175	178	171	190	241	247	150	150	161	172
RS_10	173	188	111	153	148	157	112	113	170	175	173	205	195	195	150	168	161	164
RS_11	169	183	110	113	134	136	113	119	166	179	194	226	195	195	153	153	176	179
RS_12	183	186	109	144	137	148	112	123	160	171	167	179	199	253	150	170	162	172
RS_13	169	179	152	195	134	137	119	127	166	174	188	218	193	201	150	168	164	176
RS_14	166	196	108	150	132	143	113	119	175	179	169	198	195	195	153	155	167	179
RS_15	168	194	109	120	136	136	119	125	166	183	177	202	185	199	150	152	160	162
RS_16	173	179	117	150	134	143	113	125	170	175	179	200	185	185	153	153	166	172

RS_17	168	173	113	119	134	145	123	127	166	166	171	202	195	195	152	170	162	186	
RS_18	169	211	107	192	137	140	110	113	166	175	181	198	185	237	161	172	171	171	
RS_19	169	194	108	150	134	136	110	119	179	179	162	207	237	247	161	168	165	177	
RS_20	169	173	115	144	136	143	119	119	175	175	173	179	193	207	150	153	149	176	
RS_21	173	185	113	113	132	143	119	123	160	165	175	233	193	197	153	153	154	192	
RS_22	169	180	108	109	134	134	117	123	175	175	173	181	195	195	153	167	161	179	
RS_23	169	171	109	192	140	143	116	119	170	175	164	179	?	?	?	?	?	?	
RS_24	190	190	116	144	132	140	110	119	176	178	173	179	193	193	153	176	161	176	
RS_25	171	194	117	117	143	152	119	123	170	182	188	214	185	195	150	170	170	175	
RS_26	191	213	108	111	134	143	117	119	160	166	164	186	185	199	150	153	175	175	
RS_27	177	177	113	150	145	152	119	119	160	178	186	188	185	195	172	172	149	161	
RS_28	169	197	116	142	136	140	113	119	175	178	183	214	223	241	152	152	154	177	
RS_29	171	190	108	115	134	143	122	125	175	178	183	186	185	195	152	172	175	182	
RS_30	173	191	108	112	134	134	113	119	175	178	169	220	?	?	?	?	?	?	
RS_31	177	192	153	157	134	143	110	123	175	175	177	179	195	201	150	167	149	172	
RS_32	169	173	147	147	134	148	119	119	178	179	242	242	185	201	150	153	161	165	
RS_33	179	196	108	111	143	145	113	117	170	170	186	216	193	195	153	170	174	175	
RS_34	164	183	115	192	136	140	125	125	166	166	175	179	185	201	168	172	160	165	
RS_35	164	173	107	150	136	136	119	119	179	179	169	242	185	201	172	172	153	164	
RS_36	174	177	109	150	143	157	119	123	170	174	183	194	185	203	153	153	170	179	
RS_37	190	194	107	150	134	136	117	119	178	178	171	177	195	195	153	164	161	166	
RS_38	179	185	116	195	134	140	110	113	178	178	171	186	185	191	164	164	153	167	
RS_39	176	188	111	111	136	143	119	129	166	175	171	183	195	233	164	168	153	167	
RS_40	176	191	104	108	134	140	119	123	176	176	164	175	185	185	153	153	172	176	
RS_41	186	194	108	108	143	145	113	125	166	166	173	186	185	185	150	163	167	179	
RS_42	174	190	104	111	134	136	119	123	166	175	173	186	195	195	168	168	162	175	
RS_43	188	217	142	142	136	140	119	123	166	175	173	188	191	191	150	150	153	165	
RS_44	171	173	147	150	134	140	107	125	166	178	175	177	185	195	150	152	165	170	
RS_45	171	173	150	150	134	136	119	125	178	178	177	183	195	195	153	153	175	175	
RS_46	169	196	109	147	140	140	113	119	165	165	171	179	185	185	150	152	162	192	
RS_47	171	196	109	138	136	137	119	134	166	174	160	235	185	195	172	172	167	167	
RS_48	179	179	108	108	137	165	119	127	?	?	171	218	201	227	161	170	161	177	

pop = Tern

Tern_01	169	171	108	111	134	143	113	113	165	175	179	179	195	195	153	153	152	184
Tern_02	169	169	107	107	143	145	113	113	175	175	179	179	195	195	152	152	184	184
Tern_03	169	169	107	107	143	143	113	129	175	175	179	183	195	195	150	153	165	184
Tern_04	169	169	107	109	134	143	113	113	175	175	183	183	195	195	150	153	184	190
Tern_05	169	171	107	111	134	134	113	113	175	175	183	183	195	195	153	153	186	186
Tern_06	169	171	107	109	134	143	113	113	175	175	179	179	195	195	153	153	184	190
Tern_07	?	?	?	?	?	?	113	113	175	175	179	183	195	195	150	153	184	184
Tern_08	171	171	108	108	143	143	?	?	?	?	?	?	195	195	153	153	165	184
Tern_09	171	179	107	108	134	143	113	113	175	175	171	183	195	213	153	153	184	184
Tern_10	169	171	107	107	143	143	113	113	175	175	183	183	195	195	153	153	184	184
Tern_11	169	169	111	111	134	143	113	113	175	175	179	183	150	195	150	150	184	184
Tern_12	171	171	107	107	134	143	113	113	175	175	179	179	195	195	153	153	184	184
Tern_13	169	169	107	116	134	143	113	113	175	187	179	183	195	195	150	153	184	184
Tern_14	?	?	?	?	?	?	113	113	175	175	179	183	195	195	150	150	184	184
Tern_15	169	171	107	109	134	134	113	113	176	176	179	183	195	195	150	153	184	190
Tern_16	169	169	108	112	134	134	113	113	176	176	179	183	195	195	150	153	165	190
Tern_17	169	171	107	107	134	143	113	129	176	188	179	183	195	195	150	153	186	190
Tern_18	169	179	109	111	134	143	113	113	114	175	179	183	195	213	150	153	186	186
Tern_19	171	171	107	107	134	134	113	113	175	188	179	183	195	195	150	150	186	186
Tern_20	169	171	108	109	134	143	113	113	176	176	179	179	195	195	150	153	186	190
Tern_21	171	171	108	108	143	143	113	113	176	176	179	183	195	195	150	153	186	190
Tern_22	171	171	108	109	134	143	113	113	175	175	179	183	195	213	152	152	186	186
Tern_23	169	171	108	108	134	143	113	113	176	176	179	179	195	195	153	153	167	186
Tern_24	?	?	?	?	?	?	113	125	166	176	179	183	195	195	150	153	186	186
Tern_25	171	171	108	108	134	143	113	129	165	176	179	179	195	199	153	153	186	186
Tern_26	171	171	107	107	134	143	113	113	175	175	179	183	195	213	150	153	186	190
Tern_27	169	171	107	107	134	143	113	113	175	175	179	179	195	213	152	152	186	186
Tern_28	169	169	107	109	134	143	113	113	175	175	171	183	195	213	153	153	166	184
Tern_29	169	171	109	109	134	134	113	113	176	176	179	183	195	195	153	153	186	186
Tern_30	171	171	109	109	134	143	113	113	176	176	179	179	195	195	152	152	165	190
Tern_31	171	171	107	109	134	143	113	113	176	176	183	183	195	213	150	153	186	186
Tern_32	169	169	107	109	134	134	113	113	166	176	179	183	195	195	153	153	186	186
Tern_33	171	171	109	109	134	134	113	113	176	176	179	183	195	195	150	153	186	186

Tern_34	171	171	108	108	143	143	113	129	175	175	179	183	195	195	152	155	186	186
Tern_35	169	169	107	108	143	143	113	113	176	176	179	179	195	213	150	153	186	186
Tern_36	169	171	109	109	134	143	113	176	175	175	179	183	195	195	152	152	184	188
Tern_37	171	171	107	109	143	143	113	113	114	175	179	183	195	195	152	152	186	186
Tern_38	169	185	109	150	134	137	113	113	175	175	179	179	195	195	150	152	186	186
Tern_39	171	171	109	117	143	143	?	?	?	?	?	?	195	195	150	150	186	186
Tern_40	169	169	108	108	134	143	?	?	?	?	?	?	195	195	150	153	186	186
Tern_41	169	169	108	108	134	134	113	113	175	175	179	183	195	195	150	152	186	186
Tern_42	169	171	108	109	143	143	113	113	175	175	179	183	195	195	150	152	186	186
Tern_43	169	169	108	109	140	143	113	119	175	175	179	183	195	213	150	153	184	184
Tern_44	169	171	108	111	143	143	113	113	175	175	183	183	195	213	150	153	184	184
Tern_45	169	169	108	108	136	143	113	129	175	175	179	183	195	195	150	152	186	186
Tern_46	169	171	108	109	134	134	113	113	175	175	179	183	195	195	150	150	184	184
Tern_47	169	169	108	109	134	143	113	129	175	175	179	183	195	213	150	152	164	164
Tern_48	169	169	108	109	143	143	113	129	175	175	183	183	195	195	153	153	186	186
pop = Was																		
Was_01	190	213	107	112	134	140	104	119	166	179	216	216	213	217	152	174	161	162
Was_02	?	?	?	?	?	?	?	?	?	?	?	?	?	?	152	153	?	?
Was_03	169	221	108	115	134	143	123	125	166	179	169	175	213	213	150	152	154	176
Was_04	169	169	108	109	137	140	104	119	183	183	175	238	213	217	153	174	165	167
Was_05	169	177	108	150	134	137	112	119	179	179	175	209	213	213	152	163	161	177
Was_06	168	186	108	192	137	140	117	119	178	180	181	205	213	231	150	153	155	176
Was_07	169	171	111	192	137	140	104	122	174	179	169	222	213	219	174	174	155	178
Was_08	171	179	108	115	140	143	119	119	174	179	169	181	213	233	150	150	178	178
Was_09	169	186	108	147	136	140	?	?	?	?	?	?	213	217	153	163	175	177
Was_10	183	186	108	108	136	137	119	125	179	179	175	183	195	213	152	152	171	175
Was_11	179	183	108	108	137	143	119	122	175	182	175	177	213	217	153	153	175	177
Was_12	180	196	108	193	136	140	119	125	175	175	179	181	213	237	152	163	177	177
Was_13	169	179	108	111	136	140	104	122	166	182	175	175	213	235	153	153	177	177
Was_14	213	221	108	111	134	139	104	104	166	182	175	238	213	217	150	174	175	175
Was_15	169	179	108	108	136	136	119	119	172	179	175	235	213	213	150	152	160	175
Was_16	186	188	108	193	134	143	117	119	176	176	169	194	213	237	150	150	164	184
Was_17	?	?	?	?	?	?	119	119	171	179	175	175	?	?	?	?	?	?

Was_18	169	180	107	192	137	137	119	119	179	179	169	224	213	237	153	163	176	178
Was_19	180	180	108	144	134	143	117	119	166	175	181	220	213	237	150	152	175	177
Was_20	180	217	108	193	137	140	104	119	175	175	173	175	211	211	153	153	176	177
Was_21	169	179	104	111	134	136	113	119	176	180	175	200	215	215	152	152	154	164
Was_22	188	194	115	115	137	143	119	119	?	?	169	169	195	195	153	176	161	176
Was_23	169	183	108	195	136	145	119	125	176	190	194	214	213	213	153	163	176	178
Was_24	188	194	109	192	140	143	117	119	176	180	169	207	195	213	153	153	164	172
Was_25	169	169	107	115	136	145	119	119	180	187	194	200	213	213	153	163	155	172
Was_26	180	213	193	193	136	140	119	119	167	178	169	175	213	213	153	174	175	175
Was_27	169	213	108	193	136	145	119	119	174	179	169	175	213	213	150	153	175	177
Was_28	211	211	108	108	140	143	119	125	170	179	192	198	229	237	152	163	160	175
Was_29	174	177	108	112	140	143	119	122	175	179	207	207	217	235	152	174	176	176
Was_30	169	194	108	116	137	140	113	119	175	179	188	202	213	213	150	163	160	162
Was_31	169	190	113	115	136	145	119	119	175	179	175	200	195	213	150	163	154	160
Was_32	179	213	108	109	137	137	119	125	179	179	169	179	195	213	153	153	176	179
Was_33	185	186	105	192	134	136	?	?	?	?	?	?	211	211	153	153	?	?
Was_34	169	179	108	109	137	143	119	119	175	175	169	177	211	211	150	150	161	161
Was_35	169	183	107	107	136	140	119	119	179	179	177	181	213	213	153	163	155	172
Was_36	169	213	107	107	136	140	117	119	180	187	177	202	195	213	153	163	178	180
Was_37	169	188	108	136	136	136	104	119	175	179	198	224	199	213	153	163	178	179
Was_38	169	183	107	116	136	136	119	119	176	180	169	188	213	213	153	174	155	178
Was_39	188	213	108	109	137	137	117	119	175	176	177	202	213	213	153	153	161	166
Was_40	179	213	108	108	136	140	104	104	178	179	177	218	213	235	153	178	176	176
Was_41	180	194	108	115	137	137	119	125	174	175	171	179	213	213	150	163	177	177
Was_42	186	194	108	109	136	136	104	119	182	190	177	194	213	213	152	163	161	170
Was_43	169	183	108	111	136	137	104	119	175	175	186	196	213	235	153	163	171	175
Was_44	169	183	107	115	134	134	104	119	174	179	175	205	213	213	163	174	153	164
Was_45	169	213	107	192	134	134	104	125	179	179	177	194	213	213	150	152	175	175
Was_46	169	183	116	192	136	143	104	119	174	175	175	179	213	213	150	153	178	182
Was_47	169	179	108	196	137	137	119	125	165	170	169	190	195	213	153	176	176	177
Was_48	179	186	108	193	140	145	117	122	182	182	196	216	195	213	150	153	155	178